REMARKS/ARGUMENTS

Status of the Claims

Upon entry of the present amendment, claims 27, 36-38 and 40-44 are pending. Claims 1-26, 28-35 and 39 are canceled. Claims 27 and 40 are amended.

Claim 27 is amended to set forth a method of detecting the translation of an mRNA. Support is found, for example, on page 13, line 31 through page 14, line 5 and on page 70, line 28 through page 71, line 26. Claim 27 was also amended to set forth the term "bound" in place of "docked." Support is found, for example, in Figures 14-15 and 17 and in the legends for Figures 14-15 and 17 on page 16, lines 3-15 and 20-26.

Claim 40 is amended to properly depend from claim 27.

Interview with the Examiner

Applicants thank Examiner Wessendorf for graciously granting the telephonic interview with Applicant and Applicants' attorneys on August 24, 2005. The issues discussed during the interview are set forth in the Interview Summary. During the interview, proposed language for amending independent claim 27 was presented. Examiner Wessendorf indicated that the new claim addressed the pending concerns under Section 112. The Examiner stated that an agreement as to the allowability of the claims could be reached with the submission of a Declaration under 37 C.F.R. § 1.132 to address the enablement rejection and Terminal Disclaimers to overcome the obviousness-type double patenting rejections over U.S. Patent Nos. 6,225,047; 6,579,719; and 6,881,586.

Rejection under 35 U.S.C. § 112, first paragraph, written description requirement

The Examiner has rejected claims 27 and 36-44 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. Applicants respectfully traverse this rejection, because the specification demonstrates Applicants possession of the claimed methods.

According to the MPEP, to satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail such that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. Possession may be shown in a variety of ways including description of an actual reduction to practice. Possession of a claimed invention may be demonstrated by description of the invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. MPEP § 2163(I).

The Examiner alleges that the specification does not describe a method for detecting translation of a polynucleotide from the produced polypeptide. The Examiner alleges that there is no correlation as to the polypeptide obtained to the translation of the polynucleotide. *See*, page 3 of the present Official Action. As discussed with the Examiner during the interview, this is incorrect.

As explained above, claim 27 has been amended to recite a method for detecting translation of an mRNA. Clearly, the specification describes the presently claimed methods for detecting translation of an mRNA, for example, on page 13, line 31 through page 14, line 5 and on page 70, line 28 through page 71, line 26. On page 71, lines 13-26. Applicants describe with great specificity exactly how to carry out the claimed methods. Moreover, because the methods are directed to detecting the translation of an mRNA, which does not contain introns, there is a direct correlation between the nucleotide sequence of the mRNA and the translated sequence of the polypeptide.

In the Office Action, the Examiner alleges that because of the degeneracy of the genetic code, one cannot predict whether a polypeptide is the desired product that is derived from a polynucleotide translation. *See*, page 4 of the present Official Action. As discussed with the Examiner during the telephonic interview, this is incorrect. The present methods detect the translation of a polypeptide from an mRNA sequence. Even with the degeneracy of the genetic code, the specific amino acids encoded by a particular codon in an mRNA sequence have long been known in the art. Applicants respectfully point out to the Examiner that the present methods only require detecting the polypeptide as an indicator of translation of the mRNA, and do not require determining or knowing the sequence of either the mRNA or the polypeptide.

However, if it is desirable to determine subsequently the sequence, those of skill in the art knew, at the time of filing of the present application, how to determine the sequence of an mRNA and how to predict a polypeptide sequence that would be translated from a particular mRNA sequence (*see*, Sambrook, *et al.*, *Molecular Cloning—A Laboratory Manual* (1989) and Ausubel, *et al.*, *Current Protocols in Molecular Biology*, referenced on page 71, lines 20-24 of the specification).

The Examiner is respectfully reminded that the Patent Office has the burden of providing evidence of why persons skilled in the art would not recognize that the inventors had possession of the claimed invention at the time of filing. MPEP at § 2163, *citing In re Wertheim*, 191 USPQ 90 (CCPA, 1976). The present Office Action has provided no such evidence. Therefore, in view of the above amendment and arguments, Applicants respectfully request withdrawal of the rejection for alleged lack of written description.

Rejection under 35 U.S.C. § 112, first paragraph, enablement requirement

The Examiner has rejected claims 27 and 36-44 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. This rejection is respectfully traversed, because Applicants have shown those of skill in the art how to practice the claimed methods.

In order to establish a *prima facie* case of lack of enablement, the Examiner has the burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 27 USPQ 1510, 1513 (Fed. Cir. 1993). As set forth in MPEP § 2164.01, "the test of enablement is not whether any experimentation is necessary, but whether...it is undue." The "fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation" (citations omitted). Further, a patent need not teach, and preferably omits, what is well known in the art. *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986). Finally, claims reading on inoperative embodiments are enabled if the skilled artisan understands how to avoid inoperative embodiments. *See, e.g., In re Cook and Merigold*, 169 USPQ 299, 301 (C.C.P.A. 1971).

The Examiner alleges that the specification does not teach the detection of the polynucleotide from the polypeptide produced from the translation. *See*, page 6 of the present Official Action. It appears that the Examiner has misunderstood the claims. As amended, the presently claimed methods detect *translation of an mRNA* by detecting a translated polypeptide retained on an adsorbent. The methods provide but do not detect the mRNA.

The Examiner alleges that the specification does not enable how translation of an mRNA *in situ* on a substrate can be accomplished. *See*, page 6 of the present Official Action. The present specification provides detailed guidance to those of skill in the art as to how to practice the presently claimed methods (*see*, page 70, line 29 through page 71, line 26), including step-by-step instructions for carrying out the methods for one particular embodiment, screening a phage display library (*see*, page 71, line 28 through page 77, line 13). On page 71, lines 13-26, the specification teaches with specificity how translation of an mRNA *in situ* on a substrate can be accomplished. The substrate is overlaid with a cylindrical tube, thereby creating a well. In the well one places reagents for *in vitro* translation, and the produced polypeptide binds to the adsorbent on the substrate. The cylindrical tube is removed and the adsorbent spots are washed. Retained polypeptide is subject to detection by laser desorption spectrometry.

The Examiner alleges that the specification does not describe the kind of translation reagents employed in the methods. *See*, page 6 of the present Official Action. However, protocols for carrying out *in vitro* translation and reagents for *in vitro* translation were well known in the art at the time of filing of the present application. *In vitro* translation had been practiced by those of skill in the art at least since 1972.[1] Moreover, the specification references on page 71, lines 20-24 two well known guides in the field of molecular biology, Sambrook, *et al.*, *Molecular Cloning—A Laboratory Manual* (1989) and Ausubel, *et al.*, *Current Protocols in Molecular Biology*. At the time of filing of the present invention, reagents for *in vitro* translation were readily available in kits, for instance those commercially purchasable from Promega of

[1] *See*, summary pages from results of PubMed database for "in vitro translation [title] and 1970:1997 [date]," attached as Exhibit C.

Madison, WI. Applicants attach to this response as Exhibits D-F three scientific publications disclosing the use of such kits for practicing *in vitro* translation.[2]

The Examiner has expressed concern regarding the use of expression systems in the present methods. *See*, page 7 of the present Official Action. However, although one could use an expression system in practicing the claimed methods, it is not a required step to transcribe the mRNA. The present methods set forth the step of providing an mRNA, which can, but need not involve the use of an expression system.

The Examiner has expressed concern regarding the predictability of translation of a polynucleotide. Applicants have addressed this concern by amending the claim to set forth translation of an mRNA sequence, which intrinsically is intended to be translated into a polypeptide. In addition, to confirm the operability of the present methods, Applicants submit with this response the Declaration of inventor Dr. Tai-Tung Yip under 37 C.F.R. § 1.132. An executed copy of the Declaration will be provided once it is available to the undersigned. The Declaration states that the methods can be predictably and successfully carried out without undue experimentation. The example attached to the Rule 132 Declaration demonstrates translation in an *E. coli* cell. However, mRNA can be provided via an expression system or in an already transcribed form (*e.g.*, via a commercially purchased mRNA library). The presently claimed methods set forth carrying out *in vitro* translation. Commercially purchased reagents for carrying out *in vitro* translation can be added to the well created by the cylindrical tube, and the translated polypeptide can be captured using an appropriate adsorbent.

Because Applicants teach those of skill in the art how to practice the present methods without undue experimentation, the Examiner is respectfully requested to withdraw this rejection.

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[2] *See*, page 10479 of Hedley, *et al.*, *Proc Natl Acad Sci USA* (1994) 91:10479, attached as Exhibit D; page 3435 of Larrouy, *et al.*, *Nucleic Acids Research* (1995) 23:3434, attached as Exhibit E; and page 7975 of Wirblich, *et al.*, *J Virol* (1996) 70:7974, attached as Exhibit F.

Rejections under 35 U.S.C. § 112, second paragraph

A) translating the mRNA *in situ*

The Examiner objects to step c) of claim 27, which recites "translating the mRNA *in situ* on the substrate," because it is allegedly unclear how this would be done. Applicants specification at page 71, lines 14-26 teaches how *in vitro* translation can be carried out *in situ* on an adsorbent by overlaying the substrate with a cylindrical tube to create a well with the substrate at the base of the well. In the well, one places reagents for *in vitro* translation of the mRNA.

B) docked through the adsorbent to the substrate

The Examiner objects to recitation of the term "docked" in step c) of claim 27. Applicants respectfully remind the Examiner that the patentee can be his own lexicographer. M.P.E.P. § 2111.01(III). The present specification implicitly defines "docked" to refer to covalent or non-covalent binding. In the interest of furthering prosecution, Applicant's have replaced the term "docked" with "bound" in step c) of claim 27. Support can be found, for example, in Figures 14-15 and 17 and in the legends for Figures 14-15 and 17 on page 16, lines 3-15 and 20-26.

Obviousness-Type Double Patenting

U.S. Patent No. 6,225,047

The Examiner has rejected claims 27, 36, 38 and 44 under the judicially created doctrine of obviousness-type double patenting as allegedly obvious over claims 1 and 19 of U.S. Patent No. 6,225,047. Applicants do not agree with the Examiner. However, in the interest of furthering prosecution, Applicants submit a Terminal Disclaimer with this response, thereby rendering this rejection moot.

U.S. Patent No. 5,719,060

The Examiner has rejected claim 27 under the judicially created doctrine of obviousness-type double patenting as allegedly obvious over claim 1 of U.S. Patent No. 5,719,060. This rejection is respectfully traversed because claim 1 of the '060 patent does not

disclose or suggest the required step of the present methods of translation of an mRNA *in situ* on an adsorbent.

U.S. Patent No. 6,579,719

The Examiner has rejected claims 27, 36 and 38 under the judicially created doctrine of obviousness-type double patenting as allegedly obvious over claims 1-2, 10-11 and 19 of U.S. Patent No. 6,579,719. Applicants do not agree with the Examiner. However, in the interest of furthering prosecution, Applicants submit a Terminal Disclaimer with this response, thereby rendering this rejection moot.

U.S. Patent No. 6,881586

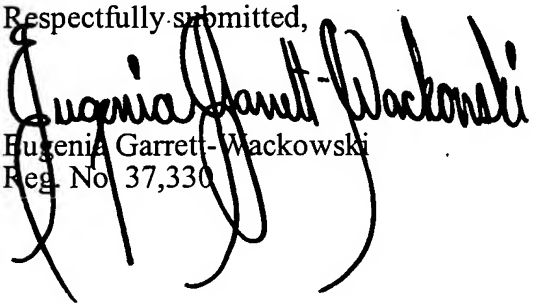
The Examiner has rejected claims 27, 36 and 38 under the judicially created doctrine of obviousness-type double patenting as allegedly obvious over claims 1-3 and 5 of U.S. Patent No. 6,881,586. Applicants do not agree with the Examiner. However, in the interest of furthering prosecution, Applicants submit a Terminal Disclaimer with this response, thereby rendering this rejection moot.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,



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Attachments
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Attachments:
Exhibit A—curriculum vitae of inventor Dr. Tai-Tung Yip, attached to Rule 132 Declaration
Exhibit B—Example showing operability of invention, attached to Rule 132 Declaration
Exhibit C—summary pages from search results of PubMed database for "in vitro translation [title] and 1970:1997 [date]
Exhibit D—page 10479 of Hedley, *et al.*, Proc Natl Acad Sci USA (1994) 91:10479
Exhibit E—page 3435 of Larrouy, *et al.*, Nucleic Acids Research (1995) 23:3434
Exhibit F—page 7975 of Wirblich, *et al.*, J Virol (1996) 70:7974

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Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10479–10483, October 1994
Immunology

Assembly and peptide binding of major histocompatibility complex class II heterodimers in an *in vitro* translation system

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Contributed by J. L. Strominger, June 3, 1994

ABSTRACT **_In vitro_ transcription/translation of HLA-DR1 cDNAs in the presence of microsomal membranes was used to study the association of major histocompatibility complex class II molecules with peptide and invariant chain (Ii) in the endoplasmic reticulum (ER). HLA-DRα and HLA-DRβ subunits assembled into SDS-unstable heterodimers in the absence of exogenous peptide. The inclusion of synthetic peptides during the α/β assembly process promoted their conversion to SDS-resistant heterodimers. Addition of Ii RNA during the translation of HLA-DRα and HLA-DRβ RNAs resulted in the formation of α/β/Ii complexes. Peptide binding by class II molecules was detected even when excess Ii was present during α/β assembly. These findings indicate that peptides can bind α/β heterodimers in the ER microenvironment and suggest that peptides derived from cytosolic proteins that are presented by class II molecules at the cell surface may have bound to HLA-DR in the ER.**

Major histocompatibility complex (MHC) class II HLA-DRα and HLA-DRβ chains assemble with each other and invariant chain (Ii) in the endoplasmic reticulum (ER). The physical association of Ii with MHC class II heterodimers is thought to block premature peptide binding, but whether this is an allosteric effect or involves steric obstruction of the peptide binding groove is not known (1–3). Once associated with Ii, MHC class II molecules are trafficked to endosomes by a targeting signal on the cytoplasmic tail of Ii (4, 5). While en route to the cell surface, Ii is proteolytically removed (6) and class II molecules then bind peptides before being transported to the cell surface.

Analysis of peptides extracted from human MHC class II molecules has identified a small percentage of HLA-DR-bound peptides that were derived from cytoplasmic source proteins (3, 7). In addition, other reports have suggested that class II molecules can bind peptides in the ER (8–12). While these data suggest that HLA-DR might bind peptide in a preendosomal compartment, the precise cellular location in which these peptides were bound has not been determined.

Upon binding most types of peptide, class II heterodimers become resistant to SDS-induced dimer cleavage and migrate as a 60-kDa complex on SDS/PAGE (13, 14). Interestingly, class II molecules expressed in Ii-deficient mice are not SDS stable (15–17), implying that these class II heterodimers are devoid of peptide. Recently, however, antigen presenting cells from Ii-deficient mice, which are also transgenic for epitopes from myelin basic protein, have been shown to present this endogenous antigen to T cells, thus demonstrating that some fraction of class II molecules in Ii° mice are peptide loaded and functional (18). Moreover, not all peptides induce the formation of SDS-stable class II molecules upon binding (19), and it is possible that the peptides available for association with class II in the ER may be of this type. Thus, data from studies on class II-restricted endogenous antigen presentation and the Ii-deficient mice have not resolved whether peptide–class II interaction can occur in the ER.

To definitively address this question, *in vitro* transcription/translation in the presence of canine pancreatic microsomal membranes was used to analyze the assembly of HLA-DRα, HLA-DRβ, and/or Ii molecules and to determine their peptide binding capabilities.

MATERIALS AND METHODS

Antibodies. DA6.147 and PIN1 were gifts from P. Cresswell (Yale University). Rabbit anti-DRβ1 antiserum was a gift from H. Ploegh (Massachusetts Institute of Technology). BU43 was a gift from I. C. M. MacLennan (Birmingham, U.K.).

Peptides. All peptides were synthesized by standard 9-fluorenylmethoxycarbonyl chemistry, purified by reversed-phase chromatography, and verified by mass spectrometry; the final concentrations were determined by quantitative amino acid analysis or weight.

Cloning. cDNAs for HLA-DRA1 and HLA-DRB1*0101 were cloned into pGEM2 (Promega) and pCITE (Novagen), respectively. The 5′ untranslated region of the HLA-DRβ cDNA was removed to improve translation efficiency. Ii cDNA was cloned into pSELECT-1 (Promega). The first ATG (Ii p35 translation start site) was mutated to ATC by site-directed mutagenesis as described by the manufacturer of the Altered Sites *in vitro* mutagenesis system (Promega).

***In Vitro* Transcription and Translation.** Plasmid DNAs were linearized with the appropriate restriction enzymes and RNA was transcribed using the ribomax T7 kit from Promega. Transcriptions were performed following the instructions provided by the manufacturer.

Translations were performed using the conventional or flexi-rabbit reticulocyte translation kits (Promega). The protocol provided by the manufacturer was followed with the addition of 2.5 μl of [^{35}S]methionine (Amersham) and 1.5 μl of canine pancreatic microsomal membranes. KCl (0.8 μl per 25 μl of reaction mixture) was added when the flexi-rabbit reticulocyte translation kit was used. When the conventional reticulocyte lysate kit was used, 1.5–2 mM oxidized glutathione was added to the translation mixtures. Translations were for 60–90 min at 37°C. Samples were spun at 14,000 × *g* for 5 min to pellet the membranes and then washed in 0.5 ml of 130 mM KCl/66 mM Tris·HCl, pH 7.5/33 mM EDTA, pH 8.0, and repelleted for 10 min. The membranes were solubilized in 40 μl of 150 mM NaCl/50 mM Tris·HCl, pH 7.5/1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride/10 mM aprotinin/10 mM leupeptin/10 mM iodoacetamide.

Immunoprecipitations. For immunoprecipitations, 35 μl of membranes was mixed with 270 μl of immunoprecipitation

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Abbreviations: MHC, major histocompatibility complex; Ii, invariant chain; ER, endoplasmic reticulum; HA, hemagglutinin; ApoB, apolipoprotein B.

†To whom reprint requests should be addressed.

(IP) buffer containing 225 mM NaCl, 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40. Purified antibody (1 mg/ml) was added to achieve a dilution of 1:500. The samples were incubated at room temperature (RT) for 1–2 hr. Protein A-Trisacryl (15 μl) and protein G-Sepharose (15 μl) 50% slurries (both preincubated in 1% bovine serum albumin for 15 min and washed in IP buffer before use) were added to the samples and incubated for 1–2 hr. Samples were washed in IP buffer four times and the beads were resuspended in 1% SDS sample loading buffer. Samples were boiled or incubated at RT for 10–60 min before being loaded onto SDS/12% polyacrylamide gels. After electrophoresis, the gels were fixed and dried. Densitometric analysis was performed on a Fuji BAS2000 bioimager.

RESULTS

***In Vitro* Translation Assay and Antibody Specificity.** cDNAs encoding Ii, HLA-DRA1, and HLA-DRB1*0101 proteins were cloned into expression vectors containing a T7 RNA polymerase promoter. To distinguish between HLA-DRα and Ii proteins by one-dimensional SDS/PAGE, the p35 alternative translation start site in the Ii cDNA was mutated so that only the p33 form of the protein was produced (see *Materials and Methods*). *In vitro* translation of the RNAs encoding HLA-DRα, HLA-DRβ, and Ii generated proteins that, based on their migration in SDS/polyacrylamide gels, were appropriately modified by signal sequence cleavage and N-linked glycosylation (data not shown).

To verify the specificity of the antibodies used in the immunoprecipitation experiments, HLA-DRα, HLA-DRβ, and Ii RNAs were translated and the resulting proteins were immunoprecipitated with a panel of antibodies. The data show that only DA6.147 precipitated HLA-DRα (Fig. 1, lanes 1–4), a rabbit antiserum developed against gel-purified HLA-DRβ chains was the only reagent to react with the HLA-DRβ chain (lanes 5–8), and PIN1 was the only antibody to recognize Ii (lanes 9–12). LB3.1, a HLA-DRα/β complex-dependent monoclonal antibody, did not recognize any of the single chains (lanes 2, 6, and 10).

Assembly of the HLA-DR Heterodimer. *In vitro* transcription/translation has been successfully used to study MHC class I assembly (20–22). In this report, a similar system was used to study α/β and α/β/Ii assembly.

HLA-DRα and HLA-DRβ RNAs were cotranslated or translated independently and the products were immunoprecipitated with the HLA-DRα-specific monoclonal antibody DA6.147 (Fig. 2). Since HLA-DRβ is coimmunoprecipitated when both chains are translated and immunoprecipitation with LB3.1, a conformation-dependent antibody, also recognized the *in vitro* translated HLA-DR1 heterodimer, suggesting that it is folded correctly (data not shown), these data clearly demonstrate that the HLA-DR1 heterodimer forms in the absence of exogenous peptide or Ii and indicate that the *in vitro* translated products assemble.

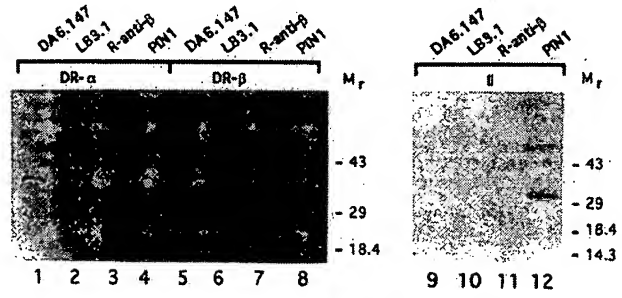

FIG. 1. Immunoprecipitation of HLA-DRα, HLA-DRβ, and Ii from *in vitro* translations. After immunoprecipitation, the products were resuspended in SDS loading buffer and analyzed by SDS/PAGE. Lanes: 1–4, HLA-DRα; 5–8, HLA-DRβ; 9–12, Ii and M_r markers (×10⁻³). Antibodies used in each immunoprecipitation are indicated above the lane. R-anti-β, rabbit HLA-DRβ antiserum.

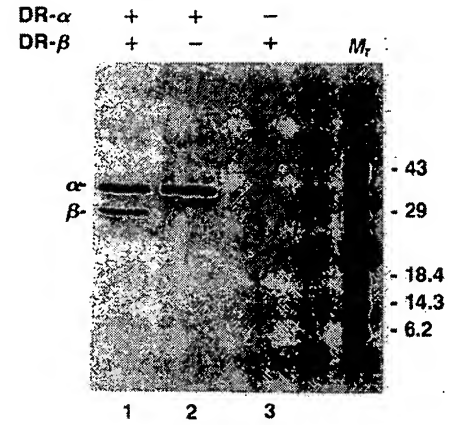

FIG. 2. *In vitro* formation of HLA-DRα and HLA-DRβ heterodimers. HLA-DRα and HLA-DRβ RNAs were cotranslated (lane 1) or translated independently (lanes 2 and 3) and immunoprecipitated with DA6.147. Lane M_r, molecular weight markers (×10⁻³).

Association of Ii with HLA-DRα and HLA-DRβ. It has been reported that Ii can associate with individual HLA-DR chains (2, 5, 23) and with assembled α/β heterodimers (24). The translation/transcription system was used to determine whether these interactions are detectable in the *in vitro* translation system. In each of these experiments the RNAs were titrated to achieve equimolar amounts of HLA-DRα and HLA-DRβ chains and a molar excess of Ii. The intensity of the immunoprecipitated bands is related to the number of methionines in each of the three proteins. The DA6.147 monoclonal antibody immunoprecipitated HLA-DRα from the α/Ii cotranslation, but Ii was not coimmunoprecipitated (Fig. 3, lane 6). PIN1 and BU43 immunoprecipitated Ii from the α/Ii mixture but did not immunoprecipitate detectable

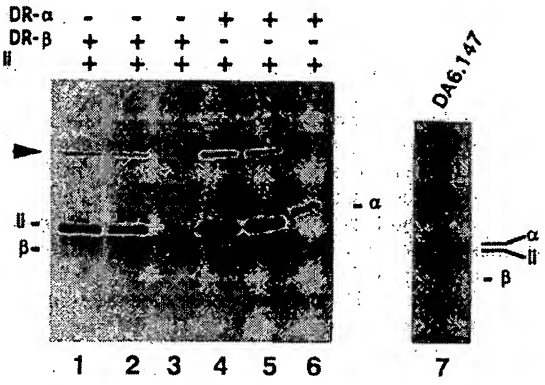

FIG. 3. Ii does not associate with HLA-DRα or HLA-DRβ chains alone but does associate with the HLA-DRα/β heterodimer. HLA-DRβ and Ii RNAs were cotranslated (lanes 1–3) and immunoprecipitated with BU43 (lane 1), PIN1 (lane 2), or rabbit HLA-DRβ antiserum (lane 3). HLA-DRα and Ii RNAs were cotranslated (lanes 4–6) and immunoprecipitated with BU43 (lane 4), PIN1 (lane 5), or DA6.147 (lane 6). HLA-DRα, HLA-DRβ, and Ii RNAs were cotranslated (lane 7) and immunoprecipitated with DA6.147. Note that the number of methionine residues in each protein is different (Ii, 15; HLA-DRα, 3; HLA-DRβ, 2), so that autoradiography of equimolar amounts of protein would reveal a band representing Ii that is stronger than the bands representing HLA-DRα or HLA-DRβ. Arrowhead indicates a putative Ii homodimer. A small amount of a slower-migrating band that may represent an Ii homotrimer is also evident.

levels of HLA-DRα (lanes 4 and 5). Both PIN1 and BU43, which recognize different domains of Ii, were used in these experiments in the event that single-chain–Ii interactions might interfere with antibody recognition. Similar results were seen when the β/Ii cotranslation was analyzed. Rabbit HLA-DRβ antiserum immunoprecipitated the HLA-DRβ chain but not Ii chain (lane 3), and the Ii-specific antibodies immunoprecipitated only Ii from the β/Ii mixture (lanes 1 and 2). Although Ii does not appear to bind single HLA-DR subunits in these experiments, the possibility that these interactions occur at levels not detectable by this assay or that the antibodies used here do not react with α/Ii or β/Ii complexes is not excluded.

The lack of detectable single-chain interactions with Ii made it feasible to use these antibodies to examine α/β/Ii assembly. When HLA-DRα, HLA-DRβ, and Ii RNAs were cotranslated and the products were analyzed by immunoprecipitation, all three proteins were coimmunoprecipitated with DA6.147 (Fig. 3, lane 7), demonstrating that α/β/Ii complexes were generated in the microsomes.

Peptide Binding to HLA-DR1. Since synthetic peptides are able to enter microsomes (20, 25, 26), and, as shown above, HLA-DR1 molecules assemble in these vesicles, experiments were done to determine whether peptide binding could occur in this microenvironment. The binding of most peptides induces stability of MHC class II heterodimers in SDS/polyacrylamide gels (13, 14). Therefore, the following assay was used to monitor HLA-DR–peptide interaction. HLA-DRα and HLA-DRβ RNAs were cotranslated in the presence of increasing amounts of an A2-like peptide (Table 1, ref. 27). The membranes were pelleted, washed extensively to remove excess peptide (peptide was diluted at least 1:1000 after the washes), solubilized in 1% Nonidet P-40, and immunoprecipitated with DA6.147. The samples were separated on SDS/polyacrylamide gels without prior boiling. In the absence of added peptide, all of the assembled HLA-DR molecules were SDS unstable; however, as the concentration of the synthetic peptide was increased (10–400 μM), a linear increase in the number of SDS-resistant molecules was observed (Fig. 4*A*).

With this assay, other HLA-DR1 binding peptides (Table 1) were tested for their ability to induce SDS stability. Several synthetic peptides conferred SDS stability to HLA-DR1 heterodimers, including fetuin, IgKappa (κ), hemagglutinin (HA), and apolipoprotein B (ApoB) peptides (Fig. 4*B*), indicating that the ability to bind HLA-DR1 in the ER microenvironment is not limited to one peptide.

Some synthetic peptides such as Ii15 and Ii24 and the HLA-DRα peptide that have previously been found associated with HLA-DR1 did not induce detectable levels of SDS-stable HLA-DR1 heterodimers when present during translation (Fig. 4*B*, lane 5; data not shown). However, when HLA-DRα and HLA-DRβ were cotranslated, the microsomes were solubilized, and peptide was then added to the reaction mixtures, the Ii15 and Ii24 peptides were able to promote the formation of SDS-stable HLA-DR molecules (Fig. 4*C*, lanes 3 and 4). Ii24 was previously shown to induce

Table 1. Peptides tested for binding to HLA-DR1

Peptide	Amino acid sequence
Ii15	KMRMATPLLMQALPM
Ii24	LPKPPKPVSKMRMATPLLMQALPM
κ	KHKVYACEVTHQGLS
DRα	APSPLPETTENVVCALGL
ApoB	IPDNLFLKSDGRIKYTLNK
HA	PKYVKQNTLKLAT
Fetuin	YKHTLNQIDSVKVWPRRP
A2-like	VGSDWRFLRGYHQYAYDG

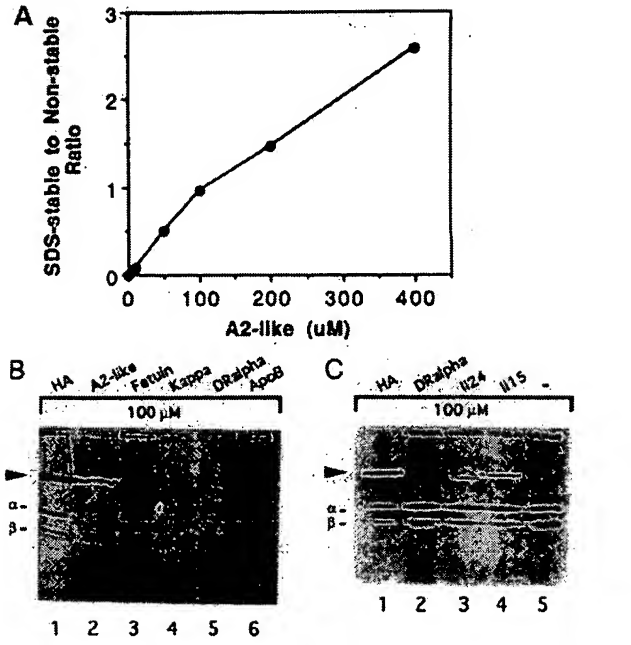

FIG. 4. Formation of SDS-stable HLA-DR heterodimers by addition of synthetic peptides. (*A*) HLA-DRα and HLA-DRβ RNAs were cotranslated in the absence or presence of A2-like peptide. Concentration of peptide in each reaction mixture is indicated on the *x* axis. The ratio of SDS-stable to -unstable heterodimers formed in each translation (*y* axis) was quantified on a Fuji BAS2000 bioimager after immunoprecipitation with DA6.147. (*B*) *In vitro* translation of HLA-DRα and HLA-DRβ RNAs was performed in the presence of 100 μM peptide. Peptide added to each translation mixture is indicated above each lane. Samples were immunoprecipitated with DA6.147 and analyzed by SDS/PAGE without prior boiling. SDS-stable heterodimer is indicated by arrowhead. (*C*) After cotranslation of HLA-DRα and HLA-DRβ, microsomes were solubilized and peptide was added at a concentration of 100 μM. Samples were incubated at 37°C for 60 min, immunoprecipitated with DA6.147, and loaded onto gels in 1% SDS sample buffer without boiling. Peptide added to each sample is indicated above the lanes; –, no peptide was added to the translation mixture. SDS-resistant form of HLA-DR is indicated by an arrowhead.

SDS stability of HLA-DR1 (27) but does not induce SDS stability of HLA-DR3 (28–30), and these differences are likely to be allotype specific. The HLA-DRα peptide did not induce SDS-stable molecules even when incubated with HLA-DR after solubilization of the membranes (Fig. 4*C*, lane 2), indicating that, although this peptide may bind HLA-DR molecules, it does not induce SDS stability in this assay. The integrity of this peptide was subsequently confirmed by HPLC analysis (data not shown).

The Effect of Ii on Peptide Binding. The data presented here demonstrate that peptides interact with HLA-DR in an ER-like microenvironment. Whether this occurs *in vivo* is likely to depend on the ability of a peptide to bind HLA-DR in the presence of Ii. The *in vitro* translation/assembly assay was used to determine whether peptides could bind HLA-DR1 in the presence of Ii. HLA-DRα, HLA-DRβ, and Ii RNAs were cotranslated in the presence or absence of 100 μM HA peptide. The interaction of class II molecules with either peptide or Ii was then assayed by immunoprecipitation. Both SDS-stable molecules (HLA-DR/peptide complexes) and α/β/Ii complexes were detected when HA was included in the translation mixture (Fig. 5, lane 2). Therefore, when Ii RNA is cotranslated with the HLA-DRα and HLA-DRβ RNAs, the HA peptide can bind HLA-DR and induce SDS-stable heterodimers (although the α/β/Ii complexes are SDS-unstable under these conditions). These data suggest

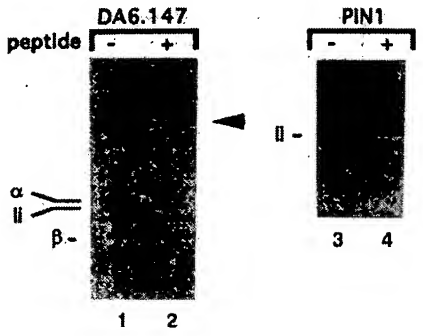

FIG. 5. Synthetic peptides induce SDS-stable HLA-DR1 dimers in the presence of cotranslated Ii. RNAs encoding HLA-DRα, HLA-DRβ, and Ii were cotranslated in the presence of 100 μM HA peptide and immunoprecipitated with DA6.147 (lanes 1 and 2). DA6.147-depleted supernatants were then immunoprecipitated with PIN1 (lanes 3 and 4). Samples were loaded onto gels in 1% SDS sample buffer without boiling.

that peptide and Ii compete for binding to HLA-DR. However, two alternative possibilities are that (*i*) Ii was limiting in the microsomes, and the non-Ii-bound class II molecules were free to interact with peptide; and (*ii*) HLA-DR1 molecules that for some reason were unable to interact with Ii were able to bind peptide. To address these possibilities the following experiments were performed.

To determine whether Ii was in excess, sequential immunoprecipitations were performed on the samples in Fig. 5. After removal of the α/β/peptide and α/β/Ii complexes with DA6.147, the samples were immunoprecipitated with PIN1 to measure the amount of excess Ii. It is clear from these experiments that free Ii is present in the translation mixtures (Fig. 5, lanes 3 and 4).

To ascertain whether two distinct populations of HLA-DR molecules were present—one that was able to interact with Ii and one that was able to bind peptide—the following experiment was done. Increasing amounts of Ii RNA were added to translation mixtures containing the A2-like or HA synthetic peptides and HLA-DRα and HLA-DRβ RNAs. As the level of Ii RNA was increased to saturating levels, the ratio of SDS-stable to non-SDS-stable heterodimers decreased (Fig. 6); however, HLA-DR1 molecules bound to peptide were still detectable. These results demonstrate that the

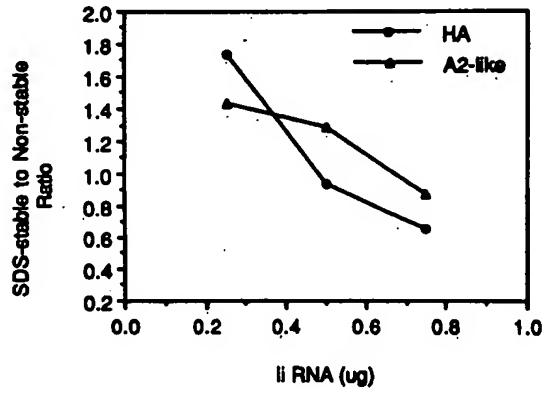

FIG. 6. Ii RNA concentration affects the level of peptide-induced SDS-stable heterodimer formation. HA (100 μM) or A2-like synthetic peptide was added during each α/β/Ii cotranslation. Amounts of HLA-DRα and HLA-DRβ RNAs were held constant but the Ii RNA concentration (x axis) was increased until HLA-DRα and HLA-DRβ translation was markedly decreased, thereby ensuring a vast excess of Ii in the microsomes. Ratio of SDS-stable to -unstable heterodimers formed in each translation mixture was quantified on a Fuji BAS2000 bioimager after immunoprecipitation (y axis).

population of HLA-DR molecules that bind peptide in the ER is likely to be the same as that which associates with Ii and suggest that, although peptides can bind HLA-DR in the presence of Ii, most of the heterodimers are associated with Ii.

DISCUSSION

The studies presented here describe an *in vitro* system designed to study peptide binding and assembly of MHC class II heterodimers. In this system, HLA-DRα, HLA-DRβ, and Ii RNAs are cotranslated in the presence of microsomal membranes that simulate the microenvironment of the ER. α/β complexes assemble in this system and bind peptides as assayed by stable heterodimer formation in SDS/PAGE. α/β/Ii complexes also assemble and synthetic peptides are able to bind HLA-DR in the presence of Ii.

Ii is thought to prevent class II proteins from binding peptide in the ER (1–3). However, most class II molecules of Ii-deficient mice are devoid of SDS-stabilizing peptides (15–17), suggesting that aside from Ii there are other reasons for the apparent inability of these class II molecules to bind peptides in the ER. These may include the following:

(*i*) The environment in the ER is not suitable for peptide binding. In fact, at least one *in vitro* study indicated that the k_{on} rate for peptide binding is enhanced at acidic pH (31). Although the low pH in the normal class II peptide loading compartments may promote class II–peptide interactions, the results presented here demonstrate that there is no physiochemical obstacle for peptide binding in the ER.

(*ii*) The requirement for a specialized peptide loading system may preclude the class II–peptide interaction in the ER. There are several hypotheses that invoke the existence of a mechanism to load peptides onto class I proteins in the ER (20, 32–35), but the efficiency with which class II molecules might be loaded by this putative system is unclear. Since peptide–class II interaction normally occurs in specialized vesicles (36–38) and not the ER, this compartment is unlikely to have the machinery required to promote class II peptide loading.

(*iii*) Calnexin and other stress-related proteins that associate with HLA-DR in the ER could retain class II molecules until assembly of the heterodimer or association with Ii occurs (39–41). Association with calnexin or other chaperones may also interfere with class II–peptide interactions.

(*iv*) MHC class I binding peptides are transported into the ER lumen primarily through the TAP transporter (26, 42). It is unclear whether the synthetic peptides used here entered the microsomes via TAP. Some studies have determined that the C-terminal residue of a peptide has an effect on its TAP-dependent transport efficiencies, but no simple motif or upper size limit has been defined that consistently makes certain peptides better substrates than others (26, 42–45). Moreover, peptides are able to gain access to the ER in the absence of TAP transporters (20, 46). For these reasons, it is not possible to predict which of the peptides used in these studies would be the most efficient at membrane translocation.

Membrane pumps that remove peptides from the ER (44), or the presence of chaperones such as GP96 and BiP which bind peptides rich in hydrophobic residues (35, 47), may decrease the effective concentration of these peptides within the ER. Given the slow rate at which HLA-DR binds peptide, the interaction would not be favored under conditions of limiting peptide. The importance of peptide concentration is underscored by results which show that higher concentrations of peptide promote an increase in the number of HLA-DR molecules that acquire SDS resistance (i.e. Fig. 4).

For the reasons listed above, there may be restraints on the type of peptide able to bind HLA-DR in the ER, but it is clear

that *in vitro* class II molecules can interact with peptides in this microenvironment. An important concern is the frequency with which this occurs *in vivo*. There are examples of HLA-DR binding to peptides derived from cytosolic proteins (3, 7, 11, 48). Given the data presented here, one interpretation for these results is that class II molecules may have acquired these cytosolic peptides in the ER. Several criteria will determine the efficiency of this process *in vivo*, the most important of these being whether a given peptide can achieve a concentration within the ER that enables it to effectively compete with Ii (10).

In addition to demonstrating that peptide binds HLA-DR in the microenvironment of the ER, this report describes an *in vitro* assay that will be a valuable tool in the analysis of peptide-facilitated class II assembly and in characterizing mutations that may affect the assembly process.

Note added in proof: A report (49) describing similar results has recently appeared.

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RNase H is responsible for the non-specific inhibition of *in vitro* translation by 2′-O-alkyl chimeric oligonucleotides: high affinity or selectivity, a dilemma to design antisense oligomers

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ABSTRACT

Ribonuclease H (RNase H) which recognizes and cleaves the RNA strand of mismatched RNA–DNA heteroduplexes can induce non-specific effects of antisense oligonucleotides. In a previous paper [Larrouy *et al.* (1992), *Gene*, 121, 189–194], we demonstrated that ODN1, a phosphodiester 15mer targeted to the AUG initiation region of α-globin mRNA, inhibited non-specifically β-globin synthesis in wheat germ extract due to RNase H-mediated cleavage of β-globin mRNA. Specificity was restored by using MP-ODN2, a methylphosphonate-phosphodiester sandwich analogue of ODN1, which limited RNase H activity on non-perfect hybrids. We report here that 2′-O-alkyl RNA-phosphodiester DNA sandwich analogues of ODN1, with the same phosphodiester window as MP-ODN2, are non-specific inhibitors of globin synthesis in wheat germ extract, whatever the substituent (methyl, allyl or butyl) on the 2′-OH. These sandwich oligomers induced the cleavage of non-target β-globin RNA sites, similarly to the unmodified parent oligomer ODN1. This is likely due to the increased affinity of 2′-O-alkyl-ODN2 chimeric oligomers for both fully and partly complementary RNA, compared to MP-ODN2. In contrast, the fully modified 2′-O-methyl analogue of ODN1 was a very effective and highly specific antisense sequence. This was ascribed to its inability (i) to induce RNA cleavage by RNase H and (ii) to physically prevent the elongation of the polypeptide chain.

INTRODUCTION

Several mechanisms have been proposed for oligonucleotide-mediated inhibition of translation or reverse transcription. One of them involves the degradation of the target RNA by RNase H (1,2). Several modifications, introduced into oligomers to reduce their sensitivity to nucleases, impaired their ability to elicit RNase H activity. This is the case with methylphosphonates (3,4), phosphoramidates (5), 2′-O-alkyl RNA (6) and alpha analogues (4,7,8). Composite oligonucleotides made of differently modified (or unmodified) parts were prepared that aimed at combining different properties within a single molecule (9–11). Mixed methylphosphonate-phosphodiester oligonucleotides were synthesized in order to yield antisense sequences resistant to exonucleases and nevertheless able to elicit RNase H activity once bound to their RNA target (10,12). Phosphoramidate-phosphorothioate sandwich oligomers were also used against An2 cyclin mRNA in injected *Xenopus* embryos (13). Successful inhibition of the Ha-*ras* gene has been reported with 2′-O-alkyl RNA phosphodiester chimeras (14). In both An2 cyclin and Ha-*ras* experiments the sandwich antisense sequences were better inhibitors than either the phosphodiester DNA parent oligomer or the homogeneously modified oligonucleotide. This was shown to be related to the nuclease sensitivity of the unmodified oligonucleotides on the one hand and was ascribed to the role likely played by RNase H in the inhibition of translation on the other hand.

RNases H from either prokaryotic or eukaryotic origin can act on mismatched RNA–DNA heteroduplexes. Therefore, RNase H can cleave non-target RNA sites exhibiting partial complementarity to the antisense oligonucleotide (15), leading to non-specific inhibition in some eukaryotic cells (16,17). In order to reduce the cleavage of non-perfect hybrids, we and others previously used methylphosphonate sandwich oligomers which led to an increased specificity of the antisense chimeric oligomer compared to the unmodified one (18–20). We had previously shown that a 15mer, with a central window of five phosphodiesters inserted between two methylphosphonate flanks, targeted to the initiation codon region of the rabbit α-globin mRNA, displayed some selectivity in the inhibition of *in vitro* globin synthesis, despite the presence, on the β-globin mRNA, of nine sites sharing more than 60% homology with the primary α-target site (19).

2′-O-Alkyl oligoribonucleotides display interesting properties for antisense use: they are nuclease resistant and do not elicit RNase H activity. In contrast to methylphosphonate the chemical modification does not introduce a chiral center and therefore 2′-O-alkyl derivatives are stereochemically pure analogues. We extended our previous work to the use of 2′-O-alkyl RNA-phos-

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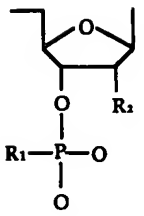

a

	Abbreviation	R_1	R_2
Methylphosphonate	MP	CH_3	H
2'-O-methyl	M	O	$O\text{-}CH_3$
2'-O-allyl	A	O	$O\text{-}CH_2\text{-}CH{=}CH_2$
2'-O-butyl	B	O	$O\text{-}(CH_2)_3\text{-}CH_3$

b

5'AUG GUG CUG UCU CCC	α-globin mRNA
TAC CAC GAC AGA GGG5'	ODN1
TAC CAC GAC AGA GGG5'	ODN2
ACA CCT TCT TCA ACC AC3'	M-ODN3
5'GG AUG GUG CUG UCU CCC CUU U	RNA [35-55]
5'G GUU AUU GUG CUG UCU CAU CAU CAU U	RNA [390]
5'GGC AAG AAG GUG CUG GCU GCC UAA	RNA [252]

Figure 1. (a) Scheme of chemical modifications used in sandwich oligonucleotides. (b) Sequences of RNA target and antisense oligonucleotides. The sequence of the initiation region of the rabbit α-globin mRNA is indicated at the top. The sense oligoribonucleotides corresponding to the perfect target [RNA (35–55)] and to the sites of highest homology on the rabbit β-globin mRNA [RNA (390) and RNA(252)] are given below, bold face characters indicate the mismatched positions. The antisense oligonucleotide ODN1 was either unmodified or prepared as the 2'-O-methyl derivative (M-ODN1). ODN2 are sandwich oligomers with methylphosphonate (MP-ODN2), 2'-O-methyl (M-ODN2), 2'-O-allyl (A-ODN2) or 2'-O-butyl (B-ODN2) residues. The underlined regions in ODN2 correspond to the modified nucleotides (either 2'-O-alkyl or 5' methylphosphonate). M-ODN3 is the 2'-O-methyl sequence complementary to nucleotides 113–129 of the β-globin message.

phodiester DNA chimeras. We demonstrate here that selectivity is not restored with such oligonucleotides which bind much more strongly to the RNA than methylphosphonate-phosphodiester oligomers. But the fully 2'-O-methyl derivative was an efficient and specific inhibitor of α-globin synthesis, indicating the key role played by RNase H in non-selective inhibition of translation by antisense oligonucleotides.

MATERIALS AND METHODS

Oligonucleotide synthesis and analysis

Unmodified oligodeoxynucleotides and methylphosphonate sandwich oligomers were synthesized on a Model 7500 Milligen Biosearch automatic synthesizer, using conventional phosphoramidite chemistry. They were purified in one step by HPLC on a reverse phase column eluted with an acetonitrile gradient (10–50%) in 100 mM ammonium acetate buffer pH 7.2. 2'-O-alkyl RNA-phosphodiester DNA sandwich oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380B using protected 2'-O-alkylribonucleoside-3'-O-phosphoramidite monomers (6) as well as standard DNA phosphoramidite monomers. All sandwich oligomers were made using an aminopropyl controlled pore glass support functionalised with 5'-O-dimethoxytrityl-3'-O-allyluridine-2'-O-succinate. Couplings were performed in the presence of tetrazole and an extended coupling time of 15 min was used. After cleavage of base labile protecting groups the crude sandwich oligonucleotides were purified 'trityl-on' by reversed phase HPLC. The trityl group was removed in the usual way and the oligomers were purified once more by reversed phase HPLC. All oligonucleotides were analyzed on a 20% polyacrylamide/7 M urea gel, following 5' end-labeling with [γ-^{32}P]ATP (37.5 TBq/mmol; NEN) and T4 polynucleotide kinase, according to standard procedures (21).

Oligoribonucleotides were synthesized by *in vitro* transcription with T7 RNA polymerase, prepared from an overproducing strain according to previously published procedures (22). Oligoribonucleotides were purified by preparative gel electrophoresis on polyacrylamide gels.

Thermal denaturation profiles of oligonucleotide hybrids were monitored at 260 nm on a Uvikon 940 spectrophotometer using a quartz cuvette of 1 cm optical path length. The temperature of the cell-holder was adjusted by circulating fluid with a Hüber Ministat cryothermostat driven by a Hüber PD410 programmer. Oligonucleotide concentrations were determined using molar extinction coefficients according to Fasman (23). RNA and DNA oligomers in a 10 mM cacodylate buffer pH 7.8 containing 50 mM NaCl were pre-heated at 100°C for 5 min. The solution was quenched on ice and magnesium acetate was added up to 1 mM. The final target RNA and antisense oligonucleotide concentrations were 1 and 2 μM, respectively.

In vitro translation

Rabbit globin mRNA (0.05 μg) purchased from Promega, was mixed with the desired oligonucleotide and added to a final volume of 30 μl of wheat germ extract (Promega), according to the supplier's instructions. Each sample contained 0.925 MBq of [^{35}S]methionine (37 TBq/mmol) from NEN. The reaction was run for 1 h at 25°C. ^{35}S-labeled proteins were analysed by electrophoresis on 15% polyacrylamide gels containing 8 mM Triton X100/8 M urea/5% acetic acid, as previously described (24). Globin synthesis was determined either from densitometer scanning of autoradiographs or from counting of gel slices. The percentage of inhibition by antisense oligonucleotides was calculated using the ratio β_i/β_0 and α_i/α_0 where α_i, α_0 and β_i, β_0 refer to the amount of α- and β-globin synthesized in the presence and in the absence of i molecules of oligonucleotide, respectively.

RNA analysis

Following *in vitro* translation under the above conditions, globin RNA was extracted with phenol and precipitated with ethanol according to standard procedures (21) and dissolved in sterile water prior to loading on a 10% polyacrylamide gel containing 7 M urea. After electroblotting the nylon membrane was probed with ^{32}P 5' end-labeled oligonucleotide complementary to nt 3–19 of the β-globin RNA. Hybridization was performed as described previously (19).

Cleavage sites on RNA-oligonucleotide heteroduplexes by the endogenous wheat germ RNase H were determined using *in vitro* transcribed oligoribonucleotides. ^{32}P 5' end-labeled oligoribonucleotide (15 nM) were incubated with oligodeoxynucleotide (1 μM) in wheat germ extract at 25°C for 1 h. Following incubation, RNA was purified as described above and analyzed on a 20% polyacrylamide/7 M urea gel. Digestion by *Escherichia coli* RNase H (1.5 U) was performed by incubating ^{32}P 5' end-labeled oligoribonucleotide/oligonucleotide hybrids, under the concentration conditions used for wheat germ, in 20 mM Tris–HCl buffer, pH 7.5 containing 10 mM $MgCl_2$, 100 mM KCl and 0.1

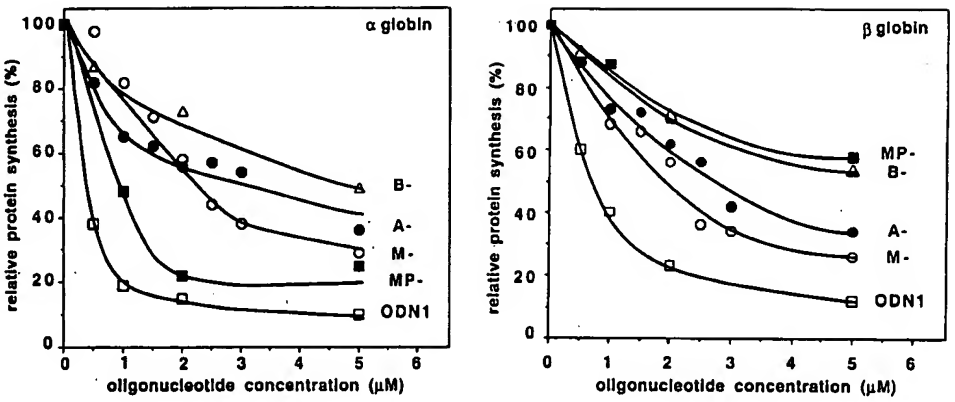

Figure 2. *In vitro* inhibition of rabbit globin synthesis by antisense oligonucleotides. Inhibition of α-(top) and β-globin (bottom) by ODN1 (□) MP-ODN2 (■), M-ODN2 (○), A-ODN2 (●) or B-ODN2 (Δ). The relative protein synthesis was calculated as described in Materials and Methods.

mM DTT. Cleavage products were analyzed on a 20% polyacrylamide/7 M urea gel. Autoradiographs were scanned by densitometry. For each sample, the relative amount of a given band was calculated with respect to all others (intact RNA and cleavage products).

RESULTS AND DISCUSSION

We targeted the nt 37–51 region of α-globin mRNA by antisense 15-mer oligonucleotides. We generated, from the complementary unmodified phosphodiester sequence ODN1, a series of chemically-modified derivatives ODN2 (Fig. 1). These oligomers, termed sandwich oligonucleotides, were made of three blocks: a central phosphodiester region, five nucleotides long inserted between flanks synthesized from nuclease resistant analogues: methylphosphonate DNA (MP), 2′-O-methyl RNA (M), 2′-O-allyl RNA (A) or 2′-O-butyl RNA (B) giving rise to MP-ODN2, M-ODN2, A-ODN2 and B-ODN2 oligonucleotides respectively (Fig. 1). These modified stretches, either methylphosphonate or 2′-O-alkyl RNA do not elicit RNase H activity. In addition we prepared fully modified 2′-O-methyl oligoribonucleotides: M-ODN1, complementary to the same region (nt 37–51) of the α-globin message and M-ODN3, targeted to nt 113–129 of the β-globin message, that is to the coding region.

Inhibition of *in vitro* translation by a 2′-O-methyl sandwich oligoribonucleotide

In wheat germ extract, the sandwich oligonucleotide M-ODN2 inhibited the synthesis of both α- and β-globin to the same extent, that is it did not display any specificity of translation inhibition (Fig. 2). Therefore, it behaved like the unmodified sequence ODN1 except that the inhibition efficiency of the 2′-O-methyl oligomer was lower than that of the parent unmodified sequence. Inhibition of β-globin synthesis can be ascribed to the presence on the β-message of several sites exhibiting partial homology, with the perfect target on the α-globin mRNA. In particular, two sites, RNA (390) and RNA (252), termed after the position of the 5′ nucleotide bound to the antisense oligonucleotide, exhibit a perfect match at 13 and 12 positions out of 15, respectively, taking into account a G.U pair in the ODN2/RNA (390) duplex (Fig. 1b).

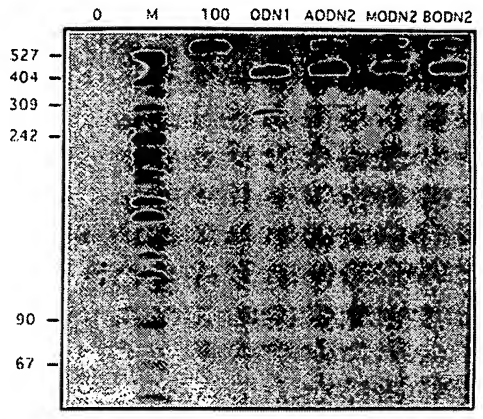

Figure 3. Northern blot analysis of rabbit β-globin mRNA following translation in wheat germ extract. *In vitro* translation was performed in the absence (100) or in the presence of 5 μM of the antisense oligonucleotide indicated at the top the lane. M refers to DNA size markers.

We have shown in a previous study, that non-specific inhibition of *in vitro* translation by the unmodified oligomer ODN1 was due to its binding to these sites, on the β-globin message (19), subsequently leading to the cleavage of β-globin mRNA by RNase H. The association of M-ODN2 with these sites and/or others might also be responsible for the non-specific effect displayed by this oligonucleotide on *in vitro* globin synthesis.

mRNA analysis after translation in the presence of a 2′-O-methyl sandwich oligoribonucleotide

Wheat germ extract is known to contain a class II RNase H activity (25). This enzyme could recognize as a substrate the mRNA region bound to the phosphodiester window of M-ODN2. Indeed, Northern blot analysis of globin mRNA revealed that both α- and β-globin messages were cleaved, following translation in wheat germ extract in the presence of M-ODN2. Two major fragments of β-globin mRNA, ~400 and 250 nucleotides long, were detected as in the case of ODN1 (Fig. 3), likely

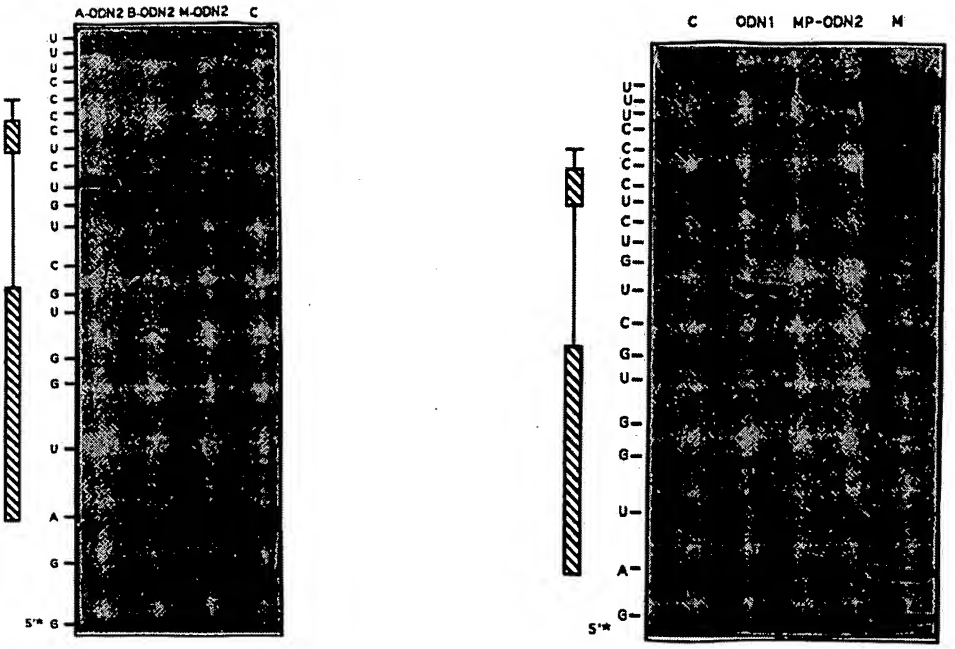

Figure 4. Cleavage analysis of antisense oligonucleotide–RNA heteroduplexes. 5′ End-labeled RNA (35–55) was incubated with the antisense oligonucleotide indicated at the top of the lane in wheat germ extract conditions used for *in vitro* translation. C corresponds to RNA (35–55) incubated in the extract, in the absence of any complementary oligonucleotide.The RNA sequence is given to the left of the panels deduced from the alkaline hydrolysis ladder (M); the boxes indicate the modified residues of the antisense sequence.

induced by the binding of antisense oligomers to the sites located at positions (390) and (252). In addition, a faint band, corresponding to an RNA fragment ~180 nucleotides long, was generated in the presence of M-ODN2 but not in the presence of ODN1 (Fig. 3). A computer search did not reveal any significant homology of this region of rabbit β-globin mRNA with the target.

It should be noted that, after *in vitro* translation intact β-globin mRNA was still observed in the presence of 5 μM M-ODN2 but not in the presence of ODN1. As the stability of the complex formed with 2′-O-methyl sandwich oligoribonucleotide, is higher than that formed with the unmodified sequence (see below), this indicated that the modified oligomer bound to RNA formed a poorer RNase H substrate than ODN1. Therefore, in contrast to the methylphosphonate oligomer MP-ODN2 (see Fig. 2 and ref. 19), the homologous sandwich oligomer M-ODN2 did not restrict RNase H activity at non-target site and, consequently, inhibited non-specifically globin synthesis in wheat germ extract.

Mapping of RNase H cleavage sites on synthetic RNA–DNA duplexes

The results obtained with MP-ODN2 on the one hand and M-ODN2 on the other hand, could be related to a different activity of RNase H on the duplexes that these oligonucleotides formed with RNA. Although the central window is identical in both sandwich oligomers, the terminal segments exhibit very different properties. The neutral internucleoside linkage of methylphosphonates or the presence of a bulky substituent on the 2′ position of 2′-O-alkyl analogues could alter either the recognition of the duplex by RNase H or the location of its catalytic site with respect to the phosphodiester stretch.

We performed a comparative analysis of a 21mer RNA [RNA (35–55)] corresponding to the target site on the α-globin message, associated either to ODN1 or to one of the sandwich oligomer, after incubation in wheat germ extract. The unmodified RNA (35–55)-ODN1 heteroduplex led to three major cleavage sites, the most prominent ones being 5–8 nucleotides away from the 3′ end of the DNA (Fig. 4). These sites were no longer seen for either of the sandwich oligomer. Instead, a strong and almost unique site appeared, shifted 3′ (with respect to the oligonucleotide) compared to the ones induced by ODN1. This position, on the 5′ side of the phosphodiester window of the sandwich oligomers, was not the same for the two types of chimeric oligonucleotides. The location of the cleavage site was close to the methylphosphonate-phosphodiester junction but was two nucleotides away from the window side for the 2′-O-alkyl analogues (Fig. 4). Surprisingly, all sandwich oligomers also induced the release of the 3′ terminal RNA residue which is four nucleotides away from the end of the heteroduplex. Therefore, this shows that the binding of the enzyme to the duplex is altered by the chemical nature of the flanks.

We then carried out a systematic investigation of the effect of both chemical modification in the antisense sequence and of mismatches, on the cleavage of heteroduplexes by *E.coli* RNase H. We used unmodified ODN1 or sandwich oligonucleotides in combination with either the perfect target RNA (35–55) or the partially complementary ones [RNA (390) and (252)]. The patterns of cleavage induced on RNA (35–55) were similar to those obtained in the wheat germ extract, except that the terminal cleavage site was not seen with sandwich oligomers MP- or M-ODN2 and that four sites instead of one were detected with MP-ODN2 (Fig. 5). The presence of mismatches led to a

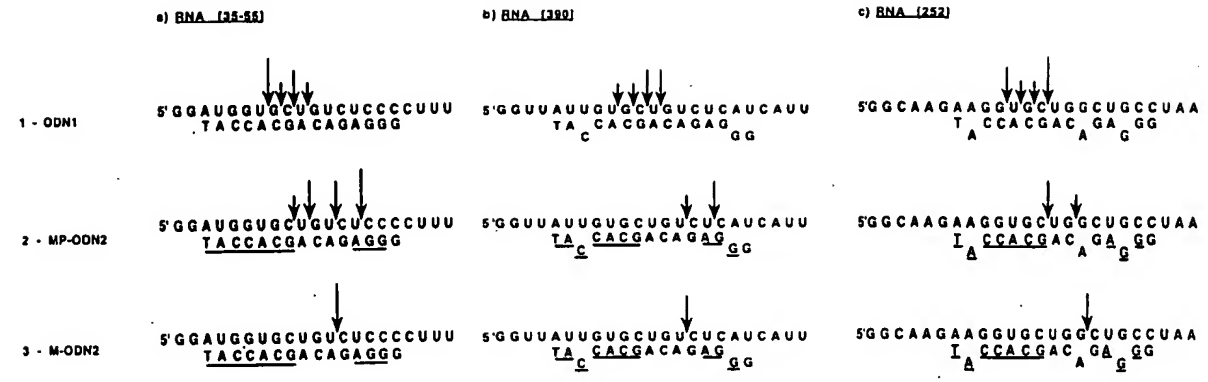

Figure 5. Location of the cleavage sites induced by *E.coli* RNase H on RNA (35–55), RNA (390) or RNA (252) in the presence of ODN1, MP-ODN2 or M-ODN2. The modified residues are underlined and mismatches are indicated. Arrows indicate both the location and the relative intensities of the cleavage sites; long, medium and short tailed arrows indicate more than 30%, between 10 and 30% or less than 10% cleavage, respectively.

moderate effect: no new cleavage site appeared, except for the ODN1/RNA(252) hybrid for which all sites were shifted by one position (in the 3′ direction with respect to the oligonucleotide). With MP-ODN2 only two out of the four sites seen with the perfect target can be seen with either of the mismatched hybrids. Identical results were obtained with M-, A- and B-ODN2 (not shown). The general trend was a reduced yield of breakdown products induced by sandwich oligomers with either RNA (390) or RNA (252) compared to RNA (35–55).

Stability of synthetic heteroduplexes

Methylphosphonate oligonucleotides bind weakly to complementary RNA sequences, compared to unmodified oligonucleotides, whereas 2′-O-methyl derivatives bind more strongly (6,26,27). We compared the binding of sandwich oligomers or of ODN1, to the perfect RNA target (35–55) and to a mismatched one corresponding to the (390) site of the β-globin mRNA. The *T*m values deduced from the UV-monitored melting curves obtained for the different mixtures are presented in Table 1. As expected, the MP-ODN2/RNA (35–55) duplex had a *T*m ~15°C lower than the ODN1/RNA (35–55) complex under our conditions. In contrast, the fully-paired hybrid formed with M-ODN2 melted at a higher temperature (ΔT = +12.5°C). The same order was observed for the stability of complexes formed with the mismatched RNA: *T*m decreased in the order M-ODN2>ODN1>MP-ODN2. In addition, MP-ODN2 led to broader transitions compared to others, indicating a lower cooperativity (not shown). This behaviour likely accounts for the differences observed between M-ODN2 and MP-ODN2. The high affinity displayed by 2′-O-methyl analogues leads to exceedingly stable duplexes, with mismatched RNA sequences, ultimately leading to non-specific effects.

Effects of other 2′-O-alkyl sandwiches

Can we achieve selective inhibition of translation with sandwich antisense oligonucleotides, made with 2′-O-alkyl segments? In order to minimize RNase H activity on mismatched hybrids we should decrease the binding constant of the antisense sequence. This could be done in two ways: (i) by reducing the length of the oligomer; or (ii) by increasing the size of the 2′-O substituent. In our case, the sequence of the (390) RNA site on the β-globin message shows the limit of the first possibility: as mismatches are close to the ends of the duplexes, shortening the antisense oligonucleotide will lead to a perfect hybrid with β-globin mRNA as well as with α-mRNA.

Therefore, we prepared sandwich antisense oligonucleotides with bulkier groups on the 2′ position as this leads to a decreased affinity for the complementary sequence (6). Indeed 2′-O-allyl (A) and 2′-O-butyl (B) analogues of ODN2 gave lower *T*m values than M-ODN2 with both the complementary sequence RNA (35–55) and with the mismatched one (Table 1). Nevertheless, the *T*m of the B-ODN2/RNA (390) duplex was still higher than the one obtained with MP-ODN2. This agrees with the fact that the trend observed with M-ODN2 was also seen with either A-ODN2 or B-ODN2: these sandwich oligomers induced non specific inhibition of β-globin synthesis (Fig. 2) and the cleavage of β-globin mRNA (Fig. 3). Moreover the same cleavage pattern was observed with short synthetic RNA (Fig. 4). Therefore in our case we were not able to achieve selective translation inhibition with chimeric oligonucleotides containing 2′-O-alkyl RNA. We then investigated the effect of the fully modified 2′-O-methyl oligomer, as no RNase H contribution to the antisense effect is expected in this case.

Table 1. Melting temperatures for antisense oligonucleotide–RNA heteroduplexes

	ODN1	MP-ODN2	B-ODN2	A-ODN2	M-ODN2	M-ODN1
RNA (35–55)	59.5	44.3	66.9	70.5	72.0	85.5
RNA (390)	41.5	36.5	44.4	49.0	50.9	63.7

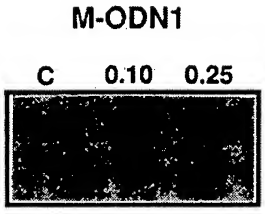

Figure 6. *In vitro* inhibition of α-globin synthesis by a 2′-O-methyl antisense oligonucleotide. Translation of rabbit globin mRNA was performed either in the absence (0) or in the presence of 0.1 or 0.25 μM of M-ODN1, the 2′-O-methyl oligoribonucleotide complementary to nt 37–51 of the α-globin message. Experimental conditions were as described in Figure 2.

Inhibition of *in vitro* translation by 2′-O-methyl oligoribonucleotides

The elongating ribosome is endowed with an unwinding activity. Consequently, antisense oligonucleotides targeted to the coding region of mRNA do not inhibit translation unless they induce permanent damage (crosslink or RNase H-mediated cleavage for instance). Indeed, M-ODN3, complementary to nt 113–129 of the β-globin message, that is to the coding region, did not induce any effect either on β-globin or on α-globin synthesis up to 5 μM (not shown). This is in fair agreement with previously published results for alpha (4,7) or 2′-O-methyl analogues (28). These derivatives do not elicit RNase H activity and cannot physically arrest the elongating ribosome.

It should be noticed that the (252) and (390) sites, partially complementary to ODN1, are located in the open reading frame of the β-message. Therefore, if RNase H is the major source of the non-specific inhibition of β-globin, in wheat germ extract, by the M-ODN2 sandwich oligomer, a fully modified 2′-O-methyl oligoribonucleotide should display a selective antisense effect towards α-globin. As a matter of fact, the 15mer M-ODN1, complementary to nt 37–51 of the α-globin mRNA induced a dose-dependent reduction of α-globin synthesis whereas no effect was seen on β-globin synthesis (Fig. 6). This is related to the RNase H-independent mechanism previously described for α-oligomers: antisense oligonucleotides targeted to the 5′ leader region, can physically impede the initiation complex to scan the mRNA (24). Therefore, the high affinity of the 2′-O-modified oligomer for its RNA target allows efficient inhibition of protein synthesis in good agreement with a recent report (28).

CONCLUSION

One of the major interests of antisense oligonucleotides is the potential selectivity which derives from the specificity of interaction between the antisense strand and the target sequence. Antisense oligonucleotides 15 nt long are expected to lead to specific inhibitory effects (29). However, RNase H which cleaves the RNA target of RNA–DNA heteroduplexes, can act on mismatched hybrids (15,30). This might be a source for non-specific effects resulting from the destruction of RNA sequences partially complementary to the antisense oligomer.

The design of sandwich molecules made of a central part allowing RNase H activity inserted between two methylphosphonate fragments, not only improved the metabolic stability of the oligonucleotides compared to unmodified ones (31), but led to increased specificity due to the restricted RNase H sensitivity of mismatched heteroduplexes (18,19). Due to the problem raised by stereo-isomers in methylphosphonate derivatives it was of interest to check whether similar properties could be displayed by other kinds of sandwich molecules.

We synthesized 2′-O-alkyl sandwich oligomers with a phosphodiester window of five nucleotides which corresponds to the lower limit to observe RNase H activity (14). We demonstrated here that the substitution of 2′-O-methyl ribonucleotides for methylphosphonate residues in a sandwich 15mer drastically changed the behaviour of chimeric antisense sequences with respect to the inhibition of translation in wheat germ extract. The methylphosphonate sandwich sequence MP-ODN2, complementary to the rabbit α-globin mRNA, displayed a higher specificity than the unmodified sequence. In contrast, none of the 2′-O-alkyl homologues of MP-ODN2 was a selective inhibitor of translation: whatever the 2′-O substituent (methyl, allyl or butyl) both α- and β-globin synthesis were decreased to the same extent. Such sandwich oligomers retained the capacity shown by the unmodified oligonucleotide, to induce the cleavage of partially complementary sites on the β-globin mRNA. The major difference between sandwich oligonucleotide–RNA hybrids resided in the stability of the various heteroduplexes. The *T*m for the duplex formed by the methylphosphonate chimeric oligomer MP-ODN2 was much lower than that obtained with the unmodified homologous antisense sequence ODN1, in agreement with previous results (32). In contrast, all 2′-O-alkyl derivatives exhibited significantly higher *T*m values. Therefore, the specificity of inhibition seems directly related to the stability of the antisense oligonucleotide-RNA hybrid and to the activity of RNase H on mismatched hybrids.

The role played by RNase H in non-specific inhibition of *in vitro* translation is further supported by the fact that fully modified 2′-O-methyl oligomer leads to a selective decrease of protein synthesis despite that it binds stronger to RNA than the sandwich oligomer. Such derivatives can prevent translation if targeted to the 5′ leader region but not to the coding region. Lastly it should be pointed out that despite their increased affinity for the RNA target, 2′-O-alkyl sandwich oligonucleotides inhibited less efficiently *in vitro* α-globin synthesis than MP-ODN2. This is likely related to a reduced ability of 2′-O-alkyl sandwich oligomers to elicit RNase H.

This work shows that exceedingly stable duplexes will give rise to non-specific effects when RNase H contributes to antisense inhibition. This will occur when long antisense sequences are used; similarly, conjugation of stabilizing ligands such as intercalating agents (29,33), will increase the affinity for both the perfect and partially complementary target sequences. The ideal antisense oligonucleotide should be chosen to saturate the target site and to simultaneously lead to negligible amounts of mismatched hybrids. Consequently the oligonucleotide should be designed with respect to the biological milieu in which it will be used. Moreover, derivatives displaying very high affinity might never allow to achieve specificity as the length of the oligomer showing the appropriate binding constant might be below the limit required to ensure the uniqueness of the target sequence in the genome of interest.

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Genetic Map of the Calicivirus Rabbit Hemorrhagic Disease Virus as Deduced from In Vitro Translation Studies

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The 7.5-kb plus-stranded genomic RNA of rabbit hemorrhagic disease virus contains two open reading frames of 7 kb (ORF1) and 351 nucleotides (ORF2) that cover nearly 99% of the genome. The aim of the present study was to identify the proteins encoded in these open reading frames. To this end, a panel of region-specific antisera was generated by immunization of rabbits with bacterially expressed fusion proteins that encompass in total 95% of the ORF1 polyprotein and almost the complete ORF2 polypeptide. The antisera were used to analyze the in vitro translation products of purified virion RNA of rabbit hemorrhagic disease virus. Our studies show that the N-terminal half of the ORF1 polyprotein is proteolytically cleaved to yield three nonstructural proteins of 16, 23, and 37 kDa (p16, p23, and p37, respectively). In addition, a cleavage product of 41 kDa which is composed of VPg and a putative nonstructural protein of approximately 30 kDa was identified. Together with the results of previous studies which identified a trypsin-like cysteine protease (TCP) of 15 kDa, a putative RNA polymerase (pol) of 58 kDa, and the major capsid protein VP60, our data establish the following gene order in ORF1: NH_2-p16-p23-p37 (helicase)-p30-VPg-TCP-pol-VP60-COOH. Immunoblot analyses showed that a minor structural protein of 10 kDa is encoded in ORF2. The data provide the first complete genetic map of a calicivirus. The map reveals a remarkable similarity between caliciviruses and picornaviruses with regard to the number and order of the genes that encode the nonstructural proteins.

The genome of rabbit hemorrhagic disease virus (RHDV), a recently identified member of the family *Caliciviridae* (10, 38, 40, 51), consists of a single plus-stranded RNA of 7,437 nucleotides that has VPg attached covalently to the 5′ end and is polyadenylated at the 3′ end. In addition to the genomic RNA, RHDV produces a subgenomic RNA of 2.2 kb that covers the 3′ third of the genome. Both the genomic and the subgenomic RNAs are packaged in nonenveloped icosahedral capsids that consist of the major structural protein VP60 (31, 32). Sequence analysis of the RHDV genome revealed an open reading frame of 7 kb (ORF1) that encodes a hypothetical polyprotein of 257 kDa. The extreme 3′ region of the RHDV genome contains a second open reading frame, of 351 nucleotides (ORF2), that overlaps ORF1 by several nucleotides and encodes a hypothetical polypeptide of 12.7 kDa. As shown by sequence comparison, the ORF1 polyprotein contains a putative RNA helicase and an RNA polymerase that are homologous to the 2C and 3D proteins of picornaviruses, respectively (31). A trypsin-like cysteine protease (TCP) of caliciviruses that is similar to the 2A and 3C proteases of picornaviruses has been identified recently (5, 54). This protease is located in the central part of the ORF1 polyprotein upstream of the putative RNA polymerase and the major capsid protein; the latter covers the C-terminal part of the ORF1 polyprotein. A similar genome organization has been reported for feline calicivirus (FCV) and two human caliciviruses, Norwalk virus and Southampton virus (7, 22, 27, 36, 37). These viruses, however, differ from RHDV in having two open reading frames of approximately 5.2 and 2 kb that encode the putative nonstructural proteins and the major capsid protein, respectively. A third open reading frame, homologous to ORF2 of RHDV, is present in the 3′ region of the genome (7, 8, 22, 27, 37).

Previous studies have shown that RHDV expresses two capsid protein species that probably differ by only two amino acids at their amino termini (5, 39, 54). The larger capsid protein, which constitutes the major structural protein in mature virions, is generated by translation of the subgenomic RNA, while the minor derivative is produced by translation of the genomic RNA and subsequent proteolytic processing at an EG dipeptide at the polymerase-capsid protein boundary. The latter cleavage occurs 2 amino acids downstream of the N-terminal methionine of the major capsid protein species and is carried out by the TCP encoded in the central part of ORF1. The TCP also exhibits self-cleavage at its N- and C-terminal boundaries. In a recent study the ORF1 polyprotein was found to be cleaved into four primary processing products, p80, p43, p73, and VP60; VP60 represents the viral capsid protein (1). The 80-kDa protein (p80) contains the 2C-like protein, while p73 encompasses the 3C-like protease and the putative polymerase.

In the present paper we report the generation of a panel of region-specific antisera that are directed against different parts of the ORF1 polyprotein and the ORF2 polypeptide. The antisera were used to study the proteolytic processing of the ORF1 polyprotein in rabbit reticulocyte lysates and to analyze the protein composition of purified virions. The results of these studies provide a complete genetic map of RHDV that assigns all of the proteins identified so far by in vitro translation, expression in bacteria, and analysis of RHDV virions to their coding sequences in the genome.

MATERIALS AND METHODS

Plasmid construction and expression in *Escherichia coli*. The pEX-34 vectors have been described previously (52, 54). Standard cloning procedures were used to construct the expression plasmids. The relevant features of the plasmids are

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TABLE 1. Expression of RHDV cDNA fragments in *E. coli*

Construct	Serum	cDNA insert[a]			Amino acids[b]		Molecular mass (kDa)[c]		Vector/restriction site(s)[d]
		Nucleotides	Restriction sites	Amino acids	RHDV	Total	Predicted	Observed	
pEX-A	A	33–918	*Bgl*I-*Nhe*I	9–303	295	431	48.4	48	A/*Bam*HI
pEX-B	B	33–344	*Bgl*I-*Nar*I	9–112	104	224	25.0	26	A/*Bam*HI
pEX-C	C	343–705	*Nar*I-*Sty*I	112–232	121	242	27.1	31	A/*Eco*RI, *Bam*HI
pEX-D	D	702–918	*Sty*I-*Nhe*I	232–303	72	203	22.8	23	B/*Eco*RI
pEX-E	E	1186–2113	*Kpn*I-*Sac*I	393–702	310	435	48	49	A/*Eco*RI
pEX-F	F	2118–3080	*Sac*I-*Eco*RI	704–1023	320	456	51	52	B/*Eco*RI
pEX-G	G	2118–2634	*Sac*I-*Bsp*HI	704–875	172	292	33.3	31	B/*Eco*RI
pEX-H	H	2631–3080	*Bsp*HI-*Eco*RI	875–1023	149	285	31.4	32	B/*Eco*RI
pEX-I	I	3077–3517	*Eco*RI-*Kpn*I	1023–1170	148	276	31.1	31	B/*Eco*RI, *Bam*HI
pEX-J	J	3522–4004	*Kpn*I-*Hin*dIII	1172–1332	161	296	32.9	33	A/*Eco*RI
pEX-K	K	4001–5189	*Hin*dIII-*Xho*I	1332–1727	396	506	56.6	55	B/*Bam*HI, *Pst*I
pEX-L	L	4001–5398	*Hin*dIII-*Bam*HI	1332–1796	465	601	67.1	66	B/*Bam*HI
pEX-M	M	5395–7047	*Bam*HI-*Bam*HI	1796–2344	549	653	69.2	72	B/*Bam*HI
pEX-N	N	7044–7437	*Eco*RI-*Eco*RI	7′–117′	111	210	23	25	B/*Eco*RI
pEX-Q		33–1181	*Bgl*I-*Kpn*I	9–390	382	504			A/*Bam*HI
pEX-R		343–1181	*Nar*I-*Kpn*I	112–390	279	395			A/*Eco*RI, *Bam*HI
pEX-S		702–1181	*Sty*I-*Kpn*I	232–390	159	275			B/*Eco*RI, *Bam*HI
pEX-T		916–1181	*Nhe*I-*Kpn*I	303–390	88	204			A/*Eco*RI, *Bam*HI

[a] Indicated are the nucleotide positions (numbered from the 5′ end of the RHDV genomic RNA) and the corresponding amino acids of the ORF1 or ORF2 (pEX-N) protein (numbered from the first methionine in ORF1 or ORF2). Except for constructs pEX-M and pEX-N, protruding ends were treated with Klenow polymerase to produce blunt ends.

[b] Indicated are the number of amino acids encoded in the RHDV cDNA fragments and the total number of amino acids of the fusion proteins.

[c] Predicted molecular mass indicates the size of the fusion proteins calculated from the amino acid sequence. Observed molecular mass indicates the apparent sizes (by SDS-PAGE analysis) of the fusion proteins. Expression of plasmids pEX-Q, pEX-R, pEX-S, and pEX-T did not yield detectable amounts of the expected fusion proteins.

[d] Indicated are the expression vectors pEX34A and pEX34B (A and B, respectively) and the restriction sites used to insert the cDNA fragments (see footnote *a*).

described in Table 1. Expression of the recombinant plasmids in *E. coli*, purification of the fusion proteins, and immunization of rabbits were carried out as previously described (53, 54).

Virus purification and RNA isolation. Rabbits experimentally infected with RHDV were sacrificed in a moribund state or shortly postmortem. The livers of the animals were removed, dissected into small pieces, and homogenized in phosphate-buffered saline (PBS) at 0°C. Large debris was removed by centrifugation in a JA 10 rotor (Beckman) for 20 min at 9,000 rpm (14,300 × *g*). The clarified supernatant was then transferred in 38-ml thin-walled centrifuge tubes (28 ml per tube), underlaid with 8 ml of 30% (wt/vol) sucrose in PBS, and centrifuged for 2.5 h at 25,000 rpm (112,000 × *g*) in a TST28 rotor (Kontron). The supernatant was removed, and the pelleted material was resuspended in PBS. For Western blot (immunoblot) analysis of viral particles, the suspension was further purified as described below. For RNA isolation, the virus suspension was treated as follows. The resuspended material was extracted several times with Freon 113. To effect phase separation, the mixture was centrifuged for 5 min at 1,000 × *g*. Viral particles in the aqueous phases were collected by centrifugation for 1.5 h at 40,000 rpm (287,000 × *g*) in a TST41 rotor (Kontron). The virions were resuspended in 2 ml of RNA lysis mix (4 M guanidine thiocyanate, 0.5% sarcosyl, 25 mM lithium citrate, 0.1 M β-mercaptoethanol), and the lysate was layered on a 2-ml cushion of 5.7 M CsCl–5 mM EDTA–10 mM Tris (pH 7.5) in a 5-ml tube and centrifuged at 36,000 rpm (164,000 × *g*) in a TST55 rotor for at least 18 h at 20°C. The pelleted RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water, precipitated with ethanol, and finally dissolved in DEPC-treated water.

Iodination of VPg. One microgram of RHDV RNA was used as the starting material for protein labeling with $Na^{125}I$ by the chloramine-T method (17). After completion of the labeling, the RNA was degraded with RNase as described before (32).

Western blot analysis of viral particles. The first steps of virus purification were carried out as described above. After the virus particles were pelleted through a 30% sucrose cushion, the particles were resuspended in PBS and layered on top of a CsCl step gradient consisting of 5 ml of a CsCl solution with a density of 1.65 g/ml and 5 ml of a CsCl solution with a density of 1.28 g/ml in PBS in a TST28 centrifuge bottle. The step gradient was centrifuged for 3 h at 22,000 rpm (87,000 × *g*) in a TST28 rotor at 20°C. After centrifugation, the tubes were punctured at the interface of the two CsCl solutions, and the virus particles were removed, dialyzed against PBS, and finally boiled in protein sample buffer. Western blot analysis was then carried out as described previously (53).

In vitro translation and immunoprecipitation. One microgram of RHDV RNA was heat denatured at 65°C for 5 min in a total volume of 10 μl, chilled on ice, and used as a template for in vitro translation in 35 μl of nuclease-treated rabbit reticulocyte lysate (RRL) (Promega) as suggested by the supplier. [^{35}S]methionine (Amersham, Braunschweig, Germany; 1,000 μCi/mmol) was used at a concentration of 1 μCi/μl to label the synthesized proteins. For immunoprecipitation analysis, the translation reaction mixture was diluted with 4 volumes of RIP buffer A (1% Triton X-100, 0.1% sodium desoxycholate, 0.1% sodium dodecyl sulfate [SDS], 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5) and 1 volume of 10% SDS. The mixture was heated at 95°C for 10 min, cooled on ice, and sonicated for 10 to 20 s. Thereafter, lysates were cleared by centrifugation for 1 h at 45,000 rpm in a TLA 45 rotor (Beckman), and the supernatant was saved for further analysis. The antisera used for immunoprecipitation generally were preincubated with 10 volumes of a 10% suspension of *Staphylococcus aureus* bacteria prepared as described by Kessler (23). The antibody-*S. aureus* mixture was incubated at room temperature for 1 h with periodic shaking; this was followed by two washes in RIP buffer A to remove unbound antibodies. The antibody-*S. aureus* mixture was then resuspended in RIP buffer A to give a 10% suspension, and 10 to 50 μl of this suspension was added to the pretreated in vitro translation mixture. After 1 h of shaking at room temperature, the bacteria were pelleted and the supernatant was either discarded or subjected to a second immunoprecipitation. The pelleted bacteria of the first and second immunoprecipitations were resuspended in RIP buffer A and layered on a 2-ml cushion of RIP buffer D (25% sucrose, 1% Triton X-100, 0.5% sodium desoxycholate, 0.1% SDS, 0.2% bovine serum albumin, 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7). The bacteria were pelleted for 15 min at 5,000 rpm at 4°C, the supernatant was removed, and the pellet was washed once with 1 ml of RIP buffer B (1% Triton X-100, 0.5% sodium desoxycholate, 0.1% SDS, 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5) and once with 1 ml of RIP buffer C (0.2% Triton X-100, 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7). The bound immune complexes were then eluted in sample buffer by heating for 10 min at 95°C. The *S. aureus* bacteria were removed by centrifugation, and the supernatants were analyzed on SDS-polyacrylamide gels as described previously (12, 47). After electrophoresis, the gels were processed for fluorography with En^{3}Hance as recommended by the supplier (New England Nuclear, Boston, Mass.), dried, and exposed to Kodak XAR-5 X-ray films at −70°C.

RESULTS

Generation of region-specific antisera. The strategy chosen to generate region-specific antisera involved the production of several fusion proteins encompassing different regions of the ORF1 polypeptide and the ORF2 polypeptide and the use of these proteins for immunization of rabbits. Table 1 and Fig. 1 summarize the relevant features of the plasmids that were constructed for the expression of RHDV cDNA fragments in

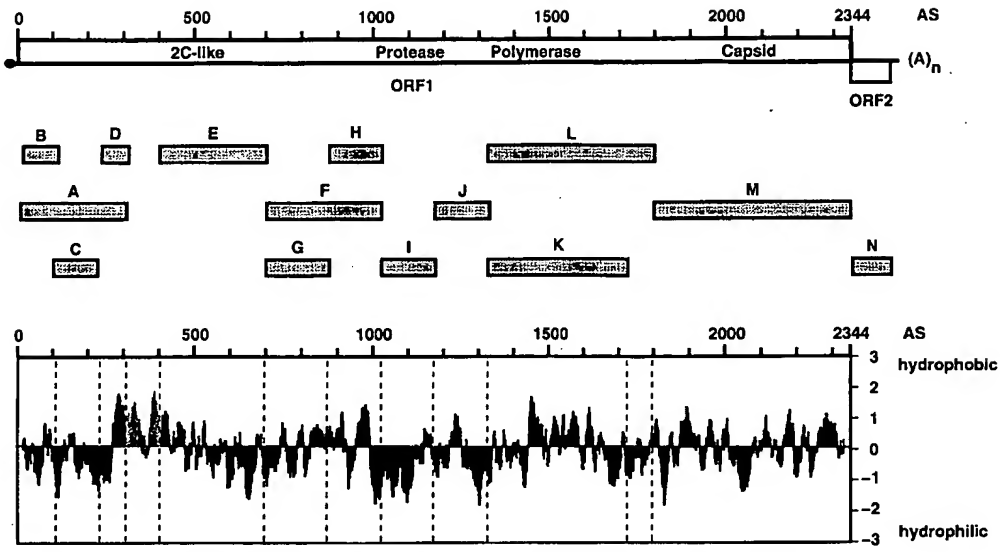

FIG. 1. Schematic representation of the RHDV genome and the cDNA fragments expressed in *E. coli*. The open reading frames ORF1 and ORF2 are indicated by bars. An amino acid scale is drawn above ORF1. The cDNA fragments A to N expressed in *E. coli* are indicated below the genome as shaded bars. The bottom part shows a hydrophilicity plot of the ORF1 polyprotein. The diagram was generated with the program PEPPLOT by using the algorithm of Kyte and Doolittle and a window size of 20 amino acids. Vertical broken lines indicate the boundaries of the polyprotein segments that are encoded by the cDNA fragments A to M. Gray shading is used to mark the part of the hydrophilicity plot corresponding to amino acids 303 to 390 of the polyprotein. This part could not be expressed in *E. coli*. AS, amino acids.

E. coli and of the fusion proteins encoded by these plasmids. In each of these plasmids, the RHDV cDNA fragment was fused in frame to the first 97 to 99 codons of the RNA polymerase gene of bacteriophage MS2; thus, each of the fusion proteins encoded by plasmids A to T comprises about 11 kDa of the MS2 polymerase in addition to the polypeptides that are specified by the RHDV cDNA fragments. Antibodies against the MS2 polypeptide could therefore be used to identify the expression products by Western blot analysis. Fusion proteins of the expected size were produced upon induction of bacteria harboring plasmids A to N, and each fusion protein was recognized by antibodies against the MS2 polymerase in Western blot analyses (data not shown). The fusion proteins appeared as insoluble aggregated material (inclusion bodies), and only a small amount of fusion protein was detected in the soluble fraction (data not shown). Highly purified fusion proteins were prepared by centrifugation of the bacterial lysate and extraction of the inclusion bodies with 7 M urea (Fig. 2). Considering expression of different parts of the ORF1 polyprotein, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the 7 M urea extract revealed an enormous variation in the fusion protein yields. The smallest amount was obtained for fusion proteins G and F.

For plasmids Q to T, fusion proteins of the expected size were not detectable in the 7 M urea extract. Western blot analyses confirmed that these proteins either were not produced or were present in very small amounts (not shown). Bacteria harboring these plasmids ceased to grow after induction, which suggested a toxic effect of the fusion proteins. Because each of the plasmids Q to T contained a short part of ORF1 from nucleotide 915 to 1185 that encodes the most hydrophobic part of the polyprotein (amino acids 303 to 390 [Fig. 1]), we suspect that this part is responsible for the toxic effect of the fusion proteins.

The fusion proteins A to N were further purified by preparative SDS-gel electrophoresis and used to immunize rabbits. The reactivities of the generated antisera were assessed by immunoprecipitation and Western blot analyses. Each of the antisera recognized its viral antigen(s), as shown below.

Location of the sequence coding for VPg. Both the genomic and subgenomic RNAs of RHDV carry a VPg of approximately 15 kDa at their 5′ ends (32). To determine the location of the coding sequence of this protein, virion RNA was labeled with radioactive iodine, and the RNA was subsequently degraded with RNase. As shown in Fig. 3, VPg was the only abundant protein in the sample (lane 11). The labeled material was subjected to immunoprecipitation with various region-spe-

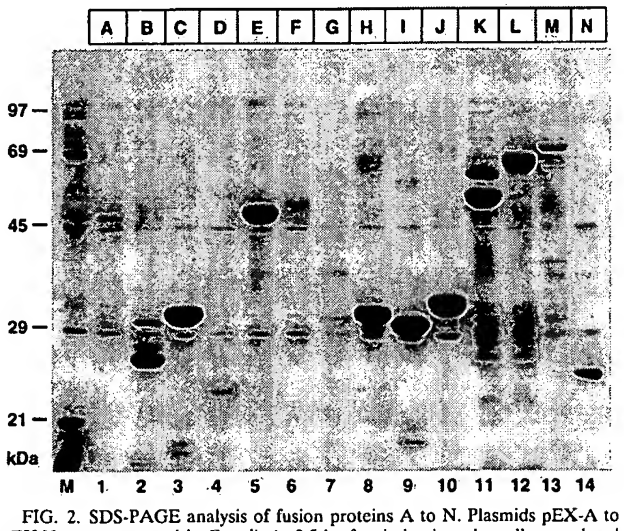

FIG. 2. SDS-PAGE analysis of fusion proteins A to N. Plasmids pEX-A to pEX-N were expressed in *E. coli*. At 2.5 h after induction, the cells were lysed and insoluble material was collected by centrifugation. The pellet was washed with 1 M urea and homogenized in 7 M urea–0.1% Tween 20. Aliquots of the 7 M urea extract were then electrophoresed in an SDS–10% polyacrylamide gel, and proteins were visualized by staining with Coomassie blue. Lane M, markers.

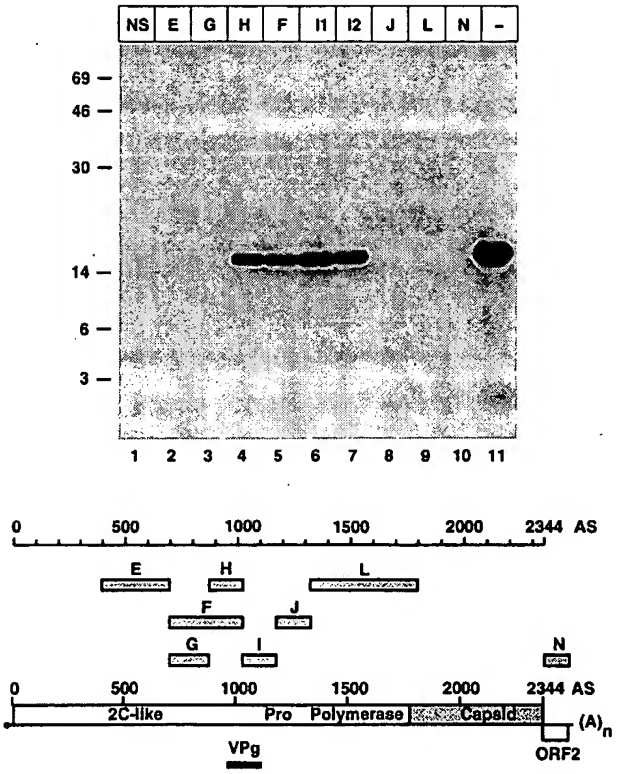

FIG. 3. Location of VPg-coding sequence. Immunoprecipitation of iodinated VPg with region-specific antisera is shown. RNA was isolated from purified virus particles and labeled with ^{125}I. The RNA was then degraded with RNase, and the radioactive material was subjected to immunoprecipitation. The RNase-digested sample (lane 11) and the immunoprecipitates (lanes 1 to 10) were electrophoresed in an SDS–12% polyacrylamide gel. Lanes I1 and I2, sera from two different rabbits which were both immunized with fusion protein I. Letters above the lanes indicate the antisera that were used for immunoprecipitation. NS, preimmune serum. A schematic diagram of the ORF1 polyprotein is shown below the gel. The position of the VPg is indicated by a black bar extending from segment H into segment I of the polyprotein. For the features of the different antisera see also Table 1.

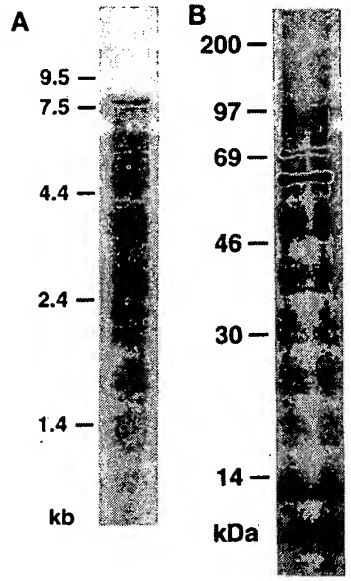

FIG. 4. Demonstration of RNA derived from RHDV virions and its in vitro translation products. (A) Northern blot with RNA isolated from gradient-purified RHDV virions hybridized against an RHDV-specific probe. The size of an RNA ladder is indicated. (B) SDS-PAGE analysis of products generated by in vitro translation of the RNA shown in panel A in the presence of [^{35}S]methionine.

cific antisera. VPg was recognized by antisera against segments F, H, and I of the polyprotein but not by antisera against segments E, G, J, L, and N (Fig. 3). This experiment showed that the VPg extends from segment H into segment I of the polyprotein.

Proteolytic processing of the ORF1 polyprotein in vitro and identification of nonstructural proteins. A tissue culture system for propagation of RHDV is not available. We therefore decided to study the proteolytic processing of the ORF1 polyprotein in vitro, using RRLs. Translation reactions were programmed with viral RNA isolated from purified viral particles. These RNA preparations contained the genomic RNA and the subgenomic RNA of RHDV (Fig. 4A). Pilot experiments confirmed our prediction that the ORF1 polyprotein of RHDV is proteolytically cleaved in the RRL, since a variety of discrete bands was detected instead of the full-length ORF1 polyprotein of about 260 kDa (Fig. 4B). To identify the cleavage products, the translation reaction mixtures were subjected to immunoprecipitation with the complete panel of region-specific antisera.

Immunoprecipitation with the anti-B serum led to the detection of one major band of 16 kDa (p16) (Fig. 5, lane 2). The same protein also reacted with the anti-A serum, which predominantly precipitated a polypeptide of 23 kDa (p23) (Fig. 5, lane 3). p23 represents the major product recognized by the anti-D serum (Fig. 5, lane 4). From these findings it was concluded that (i) p16 comprises the extreme N-terminal part of the ORF1 polyprotein and (ii) p16 most likely does not overlap p23. With regard to the molecular masses, it is probable that p23 immediately follows p16 and that the C terminus of p23 is located between segments D and E of the polyprotein. The

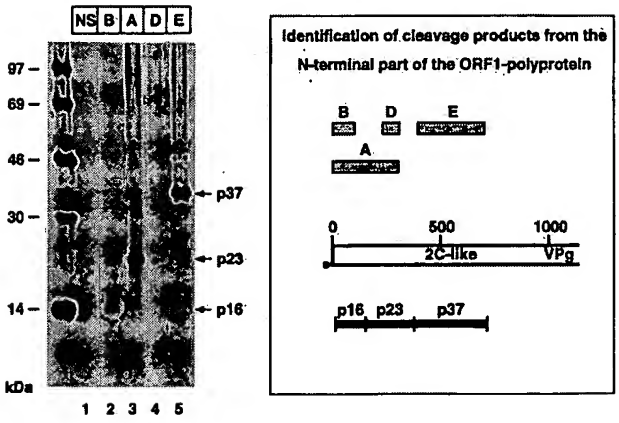

FIG. 5. Immunoprecipitation of in vitro translation products derived from the 5′ region of the RHDV RNA. RHDV RNA isolated from purified viral particles was translated in RRLs for 1 h at 30°C in the presence of [^{35}S]methionine. The translation products were identified by immunoprecipitation with the indicated region-specific antisera and SDS-PAGE analysis of the precipitated proteins. A schematic representation of the N-terminal part of the ORF1 polyprotein is shown at the right. The segments of the polyprotein that are recognized by the antisera are indicated above the polyprotein. The proteins that were precipitated by the antisera are shown as black bars below the polyprotein. The features of the antisera are summarized in Table 1.

failure to detect p23 with the anti-E serum (Fig. 5, lane 5) was consistent with this conclusion. Instead, the anti-E serum recognized a major band of 37 kDa (p37). The quantities of other bands visible in Fig. 5 varied considerably in different experiments, whereas p16, p23, and p37 were consistently detected as prominent bands. This finding indicates that these other bands probably correspond to uncleaved products encompassing two final products (i.e., bands in the range of 60 kDa) or products of internal initiation, which was always observed after in vitro translation of RHDV RNA. Taken together, the above-described data showed that the N-terminal third of the ORF1 polyprotein is processed in vitro into three major proteins of 16, 23, and 37 kDa which are arranged in the order NH_2-p16-p23-p37-. . .-COOH.

To identify the proteins derived from the central part of the polyprotein, antisera against regions F, G, H, I, and J were used (Fig. 6A). Immunoprecipitation with the anti-G serum revealed a strong band of 41 kDa, which was also detected by the anti-F, anti-H, and anti-I sera but not by the anti-J serum. This result demonstrated that p41 covers part of segment I but does not reach into segment J of the polyprotein. In addition to p41, the antisera recognized several proteins with higher molecular masses. A protein of 117 kDa (p117) was detected by all five antisera, and a protein of 87 kDa (p87) was detected by all antisera except the anti-G serum. In some lanes of Fig. 6A the latter two proteins are barely visible, because a substantial smear of translation products occurred in the high-molecular-mass range. This smear was particularly prominent in immunoprecipitations with the anti-F and anti-G sera but was also observed with other antisera. To reduce this background, a modified immunoprecipitation procedure that consisted of two sequential immunoprecipitations with different antisera was employed (Fig. 6B). Immunoprecipitation with the anti-F serum resulted again in an intense background smear that completely obscured p87 and p117, whereas p41 was still visible as a distinct band (Fig. 6B, lane 2). When two sequential immunoprecipitations were performed with anti-E as the first serum and anti-F as the second serum, both p87 and p117 were clearly visible as distinct bands (Fig. 6B, lane 3). Obviously, most of the high-molecular-mass smear was removed by the first immunoprecipitation with the anti-E serum, whereas p87 and p117 remained in the supernatant and were precipitated by the anti-F serum in the second round of immunoprecipitation. A comparison of lanes 2 and 3 of Fig. 6B revealed that the immunoprecipitation with anti-E serum also removed a 37-kDa band which was detected as a faint band by the anti-F serum in lane 2. This finding indicates that the 37-kDa band is identical to p37 described above. Taking together the results from Fig. 6A and B, the following conclusions can be drawn. (i) p41 and p117 probably possess the same N terminus, because both are recognized by the anti-G serum but not by the anti-E serum. (ii) There is probably no additional cleavage product between these proteins and the cleavage product p37, which implies that p41 immediately follows p37 in the ORF1 polyprotein. (iii) p87 lacks the N-terminal region of p41 and p117, since it is not recognized by the anti-G serum. (iv) p87 overlaps the C-terminal part of p41, since both proteins are recognized by the anti-F and anti-I sera. (v) p41 does not contain the protease and most likely ends with amino acid 1108, which represents the P1 position of a previously determined cleavage site (54). (vi) Finally, p41 contains one cleavage site and is therefore composed of two proteins. One of these proteins is represented by VPg, which extends from region H into region I of the polyprotein and covers the C-terminal third of p41 (Fig. 3). The other protein covers the N-terminal two-thirds of p41 and encompasses the region between p37 and VPg. On the

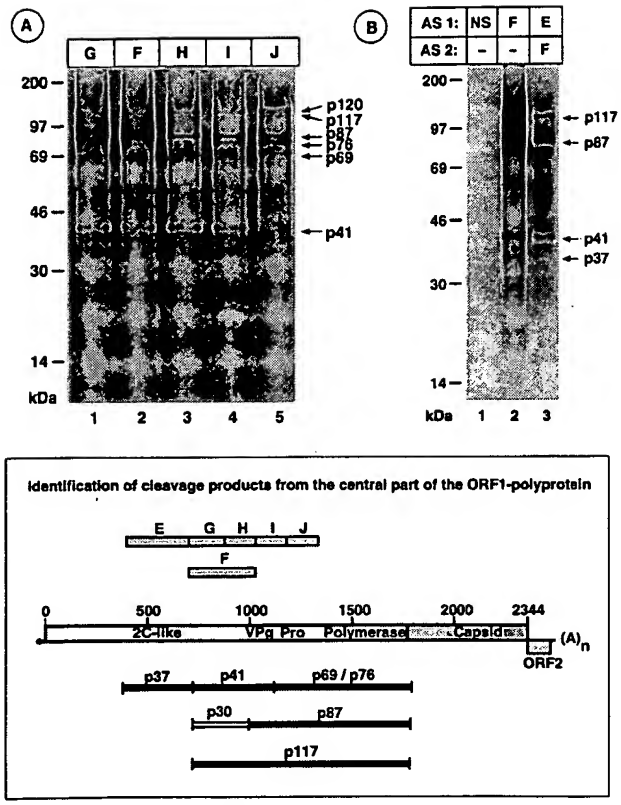

FIG. 6. Proteins encoded in the central region of the RHDV RNA. RHDV RNA isolated from purified viral particles was translated in RRLs for 1 h at 30°C in the presence of [^{35}S]methionine. The translation products were then identified by immunoprecipitation with the indicated region-specific antisera and SDS-PAGE analysis of the precipitated proteins. In one experiment the translation mixture was first subjected to immunoprecipitation with the anti-E serum, and the supernatant obtained was then subjected to immunoprecipitation with the anti-F serum (panel B, lane 3). Lane 2 in panel B shows the proteins that were recognized by the anti-F serum without prior precipitation with the anti-E serum. The dashes indicate that no second immunoprecipitation was performed. NS, preimmune serum. A schematic representation of the ORF1 polyprotein is shown below the gels. The locations of the precipitated proteins are indicated below the polyprotein, and the segments of the polyprotein that are recognized by the antisera are indicated above the polyprotein. For features of the antisera, see also Table 1. AS 1 and AS 2, antisera for the first and second precipitations, respectively.

basis of the molecular masses of p87 and p117 and assuming that both proteins have identical C termini, the postulated cleavage product that resides in the N-terminal parts of p117 and p41 has an estimated size of 30 kDa. From now on this protein will be referred to as p30. It should be noted, however, that neither p30 nor VPg was detected after in vitro translation. As shown by the formation of p87, the cleavage site at the boundary of these proteins is recognized in the RRL, suggesting that the VPg and the preceding nonstructural protein are unstable in the RRL. Protein p41 is most likely identical to the previously described p43 (1).

The data outlined above show that p41 extends to the N-terminal boundary of the protease, which is located in segment I of the polyprotein. It was therefore anticipated that the cleavage product that follows p41 should be recognized by the anti-I and anti-J sera but not by the anti-H serum. Figure 6A reveals two translation products, of 69 and 76 kDa, that satisfy these criteria. The larger protein, p76, was readily visible after immunoprecipitations with the anti-I serum, whereas the smaller

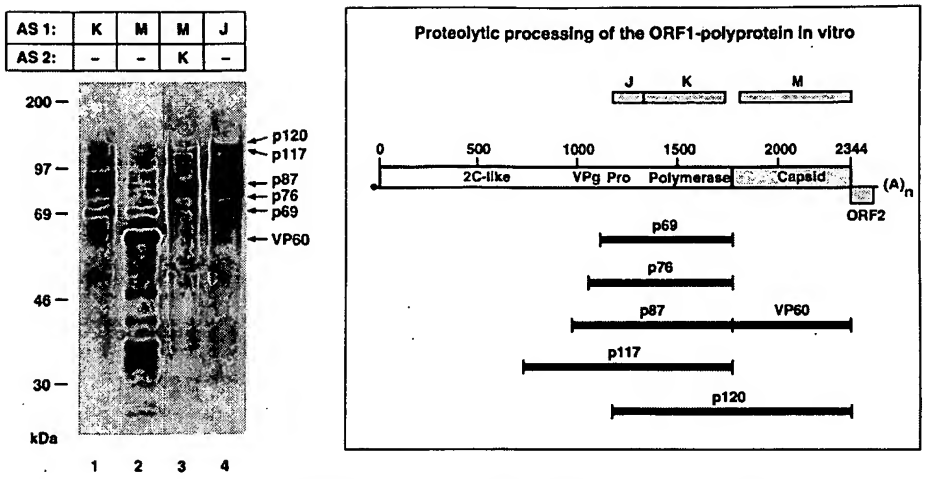

FIG. 7. Processing products derived from the 3' half of the ORF1 polyprotein. RHDV RNA was translated in RRLs and analyzed by immunoprecipitation as described for Figs. 5 and 6. The antisera used are indicated above the gel. The right part of the figure shows a schematic representation of the ORF1 polyprotein. The segments of the polyprotein that are recognized by the antisera are indicated above the polyprotein, and the locations of the precipitated proteins are indicated below the polyprotein. AS 1 and AS 2, antisera for the first and second precipitations, respectively.

protein, p69, was barely detectable with this antiserum, suggesting that p69 is not part of segment I. Both proteins extend beyond the C terminus of the protease, which is 15 kDa in size (54), and contain at least part of the polymerase. This is also the case for the cleavage products p87, p117, and a protein of 120 kDa (p120), the largest protein recognized by the anti-J serum (Fig. 7, lane 4). Additional proteins extending into the putative polymerase were detected by the anti-K serum (Fig. 7, lane 1). Since these proteins were 68 to 120 kDa in size, which is larger than the calculated molecular mass of the polymerase (58 kDa), it was concluded that most or all of these proteins extend beyond the C terminus of the polymerase into the capsid protein. This conclusion was supported by immunoprecipitation with antiserum against fusion protein M, which covers the C-terminal 58 kDa of the ORF1 polyprotein. As expected, this antiserum precipitated a major band of 60 kDa, which corresponds to the mature capsid protein VP60 (Fig. 7, lane 2). In addition, the anti-M serum precipitated several smaller proteins; these are obviously truncated derivatives of the capsid protein, since none of them was recognized by the anti-K serum. The anti-M serum also precipitated a large number of protein species with molecular masses of between 68 and 120 kDa, which are apparently due to incomplete cleavage at the polymerase-capsid protein boundary. These proteins were also recognized by the anti-K serum, which hampered identification of translation products that are cleaved at the C-terminal boundary of the polymerase. To overcome this difficulty, sequential immunoprecipitations with the anti-M and anti-K sera were performed. In such an experiment, p69, p76, p87, and p117 were specifically recognized by the anti-K serum (Fig. 7, lane 3). From this result it was clear that these proteins do not extend into the capsid protein, since otherwise they would have been removed by the anti-M serum in the first immunoprecipitation. We therefore conclude that p69, p76, p87, and p117 are generated by a specific cleavage at the C-terminal boundary of the polymerase. It should be noted that precursors comprising the capsid protein and any of these four proteins were not observed, which indicates complete cleavage at the polymerase-VP60 boundary. This is in marked contrast to the incomplete cleavage of the translation products with molecular masses of between 68 and 120 kDa, which were detected by the anti-M serum. The results from Fig. 6 show that the anti-I serum recognized p69 but not p120. This indicates that p120 and the smaller translation products, which extend from the polymerase into the capsid protein, do not include the complete protease. Accordingly, cleavage at the polymerase-VP60 boundary appears to be incomplete only for precursors that lack an active protease. Since previous studies have shown that the TCP is responsible for cleavage at the polymerase-VP60 boundary (5, 54), our data imply that at least in vitro this cleavage occurs predominantly in *cis* and is rather inefficient in *trans*. Our data also indicate inefficient cleavage at the protease-polymerase boundary, since neither the mature protease nor the mature polymerase was detected by the anti-J and anti-K sera. This finding is in agreement with our previous data which showed that the protease-polymerase boundary appears to be resistant to cleavage in vitro and is cleaved very inefficiently in *E. coli* (5, 54).

As discussed above, the 69-kDa protein is the smallest protein recognized by the anti-K and anti-J sera and also appears to be the smallest protein that includes the complete protease and the complete polymerase. From this finding it can be concluded that p69 most likely represents the cleavage product that covers the region between p41 and the capsid protein VP60 and thus is equivalent to the previously identified p73 (1). In contrast to p69, p76 probably extends beyond the N terminus of the protease into VPg and is most likely due to internal initiation. Internal initiation probably also accounts for the additional translation products, whose N termini are located within or downstream of the protease and which are not cleaved at the polymerase-VP60 boundary.

Genetic map of ORF1. The data from the in vitro translation study are summarized in Fig. 8, which assigns the products generated by in vitro cleavage of the ORF1 polyprotein to their coding sequences in the genome. The map reveals a total of seven cleavage sites, which are cleaved in vitro to different extents. At sites 1 and 3 cleavage appears to be complete as indicated by the absence of uncleaved precursor proteins that encompass the flanking cleavage products. Cleavage between the putative polymerase and the capsid protein VP60 (site 7) is also complete, taking into account that the observed uncleaved proteins are most likely aberrant products due to internal ini-

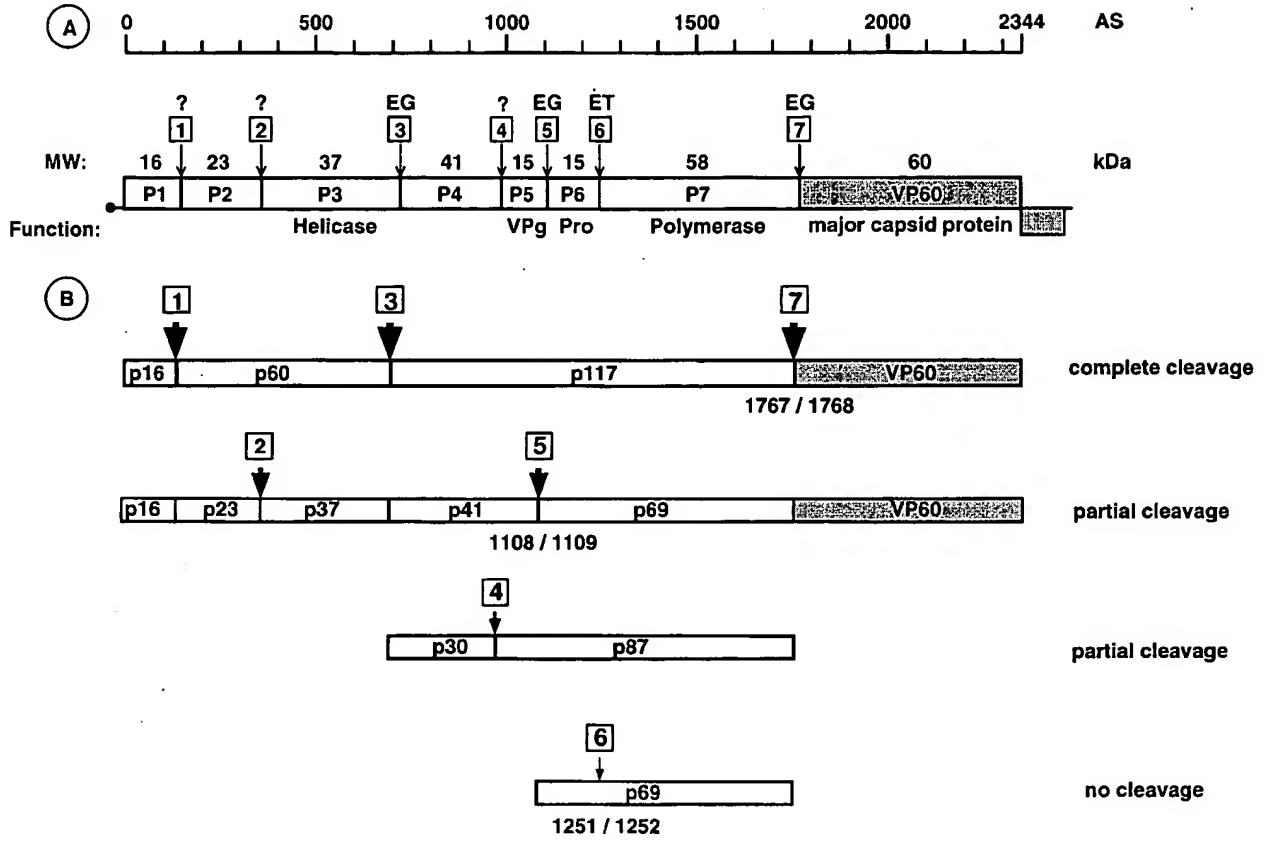

FIG. 8. Schematic representation of the in vitro cleavage products of the ORF1 polyprotein and proposed genetic map of ORF1. (A) The genomic RNA of RHDV is represented below the scale bar. Open reading frames are shown as open or shaded bars. Cleavage sites in the ORF1 polyprotein are indicated by vertical lines and numbered 1 to 7. The nonstructural proteins are designated P1 to P7. The molecular masses of the proteins (in kilodaltons) are shown above the bars, and their established or putative functions are indicated below the bars. Protein p60 was detected in several experiments but is difficult to see in Fig. 5. P4 (p41) is equivalent to a protein of 43 kDa described before (1). Cleavage sites 3, 5, 6, and 7 have been determined by N-terminal sequencing of the respective proteins or in vitro mutagenesis experiments (1, 54). Cleavage sites equivalent to sites 3 and 4 were also determined for the human calicivirus Southampton virus (29). (B) Schematic representation of the cleavage products of the ORF1 polyprotein observed in the RRL.

tiation. The in vitro assays revealed partial cleavage at sites 4 and 5 and no cleavage at site 6. In addition, several experiments indicated incomplete cleavage at site 2, since polypeptides of about 60 kDa which reacted with antisera against segments A, C, D, and E, but not B, were detected (see also Fig. 5). Although these results might in part be due to limitations of the in vitro system, it is reasonable to assume that the large variation in cleavage efficiency at the different cleavage sites reflects a mechanism by which the amount of distinct functional proteins is regulated during the viral life cycle.

The seven cleavage sites identified so far in the ORF1 polyprotein define the boundaries of eight putative final cleavage products. In our study four of these cleavage products were observed in vitro (p16, p23, p37, and VP60), whereas the remaining proteins (p30, VPg, TCP, and pol) were detected only as parts of larger proteins that are composed of two (p41 = p30 + VPg, p69 = TCP + pol), three (p87 = VPg + TCP + pol), or four (p117 = p41 + p69) of the postulated end products. Because of the limitations of our in vitro system, pulse-chase studies could not be performed. It is therefore presently unclear if the larger proteins are actually precursors that are further cleaved or if they represent stable end products. Proper cleavage of these proteins may require the presence of cellular cofactors that are lacking in the in vitro system. The failure to detect the final cleavage products may also be due to proteolytic degradation of these proteins.

The ORF2 product represents a structural protein. Viral particles were purified from a liver homogenate of an infected rabbit and subjected to Western blot analyses with region-specific antisera to examine if the virions contain viral proteins in addition to the capsid protein VP60. As expected, the anti-M and the anti-L sera showed a strong reaction with VP60 (Fig. 9). These antisera also detected smaller proteins that are commonly observed in immunoblots of RHDV particles and are probably due to proteolytic degradation of the capsid protein. No specific signal was obtained with antisera against those regions of the ORF1 polyprotein that precede the capsid protein, indicating that the proteins derived from this part of the polyprotein are nonstructural proteins or, like VPg, are present in very small amounts. In contrast, both of the anti-N sera showed a specific reaction with a protein of approximately 10 kDa. This finding indicated that the ORF2 polypeptide or a cleavage product of this protein represents a component of RHDV virions. The signal of the 10-kDa protein was much weaker than the signal of the capsid protein VP60 obtained with the anti-M serum, suggesting that the 10-kDa protein was present in a smaller amount than VP60. These results could be verified by immunoprecipitation experiments with extracts prepared after iodine labeling of purified virions. In addition to VP60, VP10 and VPg were detected. The latter two proteins were present in much smaller amounts than VP60 (data not shown). In conclusion, the analyses show that a minor capsid

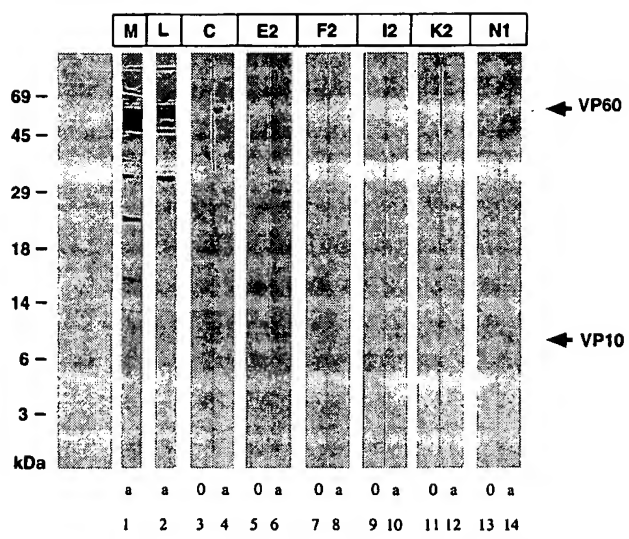

FIG. 9. Immunoblot of purified viral particles of RHDV with region-specific antisera. Purified RHDV particles were electrophoresed in SDS–12% polyacrylamide gels and analyzed by immunoblotting with the indicated region-specific antisera. Numbers are used to distinguish between sera from different rabbits. The rabbit sera were used at a dilution of 1:200. Abbreviations: a, antiserum; 0, preimmune serum. Because of the large amount of VP60 present on the blot, some of the sera show a nonspecific reaction with this protein. The features of the antisera are summarized in Table 1.

protein of approximately 10 kDa is encoded in ORF2. Furthermore, these analyses establish that VP60 and VPg are the only structural proteins encoded in ORF1.

DISCUSSION

Sequence comparisons have shown that caliciviruses are related to members of the picornavirus-like superfamily of plus-stranded RNA viruses (31, 36). This finding is confirmed and extended in our present study, which reveals a remarkable similarity in genome organization between RHDV and picornaviruses (Fig. 10). According to the results of our in vitro studies, RHDV, like picornaviruses, encodes six nonstructural proteins and VPg as parts of one large polyprotein. The nonstructural proteins include a putative RNA helicase, a TCP, and an RNA polymerase that are conserved in all members of the picornavirus-like superfamily of plus-stranded RNA viruses (11, 15, 24). In the ORF1 polyprotein of RHDV, these proteins are arranged in an order that is common to all members of the picornavirus superfamily, namely, helicase-VPg-TCP-polymerase (Fig. 10). Our data also show that RHDV encodes an additional nonstructural protein that is located between the helicase and VPg and is analogous in location to the 3A protein of poliovirus and a protein of 6 kDa encoded by potyviruses (44, 45). The N-terminal part of the ORF1 polyprotein of RHDV that precedes the putative helicase comprises the remaining two nonstructural proteins of RHDV, p16 and p23. These proteins are analogous in location to the 2A and 2B proteins of picornaviruses. Thus, each of the nonstructural proteins encoded by all picornaviruses has its counterpart in the ORF1 polyprotein of RHDV.

Previous studies showed that the TCP of RHDV is similar in function to the picornaviral 3C protease (5, 54). Although functional studies have not yet been performed for the other nonstructural proteins, the extensive sequence similarities between the putative RNA helicase 2C of picornaviruses and the nonstructural protein p37 of RHDV indicates that these proteins have a similar role in genome replication. The same argument applies to the 3D polymerase protein of picornaviruses and the RHDV RNA polymerase, the putative cleavage product p58 which constitutes the C-terminal part of the TCP-pol precursor p69. Functional homology is also obvious in the case of VPg. In picornaviruses this protein or its precursor 3AB has been proposed to serve as a primer for the viral polymerase during initiation of positive-strand RNA synthesis (14, 26, 41–43). This could also be the case for the VPgs of RHDV and other caliciviruses. It has to be stressed, however, that calicivirus VPgs are almost five times the size of picornavirus VPgs, whereas the putative helicases and RNA polymerases of RHDV and picornaviruses are very similar in size. This finding suggests that the calicivirus VPgs have additional functions that are not shared by their picornavirus counterparts. One of these functions might be to play a role in translation initiation, a process which obviously differs for calici- and picornaviruses.

While the putative helicase, the putative RNA polymerase, and, to a lesser extent, the TCP have significant sequence homology with the corresponding picornavirus proteins, this is not the case for the nonstructural proteins p23 and p30. However, our studies reveal a similar behavior of these proteins and their picornavirus counterparts in *E. coli*; like 2B and 3A of poliovirus, both p23 and p30 appear to be toxic. In contrast, the other nonstructural proteins of RHDV and poliovirus, including the trypsin-like proteases, exhibit no or only a modest toxic effect in *E. coli* (25). The toxicity of the 2B and 3A proteins of poliovirus was attributed to amphipathic helices, which are thought to interact with the bacterial membrane. Computer analysis of the ORF1 polypeptide of RHDV showed the presence of similar amphipathic helices in the N- and C-terminal parts of p23 and the amino-terminal half of p30 (data not shown). Although at present the existence of the amphipathic helices is still hypothetical for both the poliovirus polyprotein and the ORF1 polyprotein of RHDV, our results suggest that the nonstructural proteins p23 and p30 have structural and maybe also functional similarities with 2B and 3A of picornaviruses.

The extreme N-terminal part of the ORF1 polyprotein of RHDV contains a nonstructural protein of 16 kDa (p16) that occupies a position in the polyprotein analogous to the 2A protein of picornaviruses. Like p23 and p30, the nonstructural protein p16 shows no obvious sequence similarity with its picornavirus-counterpart. This is not surprising, since in picornaviruses the 2A protein is one of the most variable nonstructural proteins with regard to size and sequence. In foot-and-mouth disease virus and enteroviruses, 2A exhibits proteolytic activity and mediates proteolytic cleavage at its own N or C terminus (43). However, while in enteroviruses the 2A protease belongs to the trypsin-like family of proteases, the 2A protein of foot-and-mouth disease virus appears to be only 16 amino acids in length and shows no significant similarity to any known protease family (46). Sequence analysis of the nonstructural protein p16 did not reveal the presence of a catalytic triad typical of trypsin-like proteases. In many viral polyproteins, papain-like proteases constitute the first polypeptide (13, 16), but p16 also shows no homology to these proteases. A low level of similarity between the C-terminal part of p16 and the 2A protein of foot-and-mouth disease virus was apparent (data not shown). Preliminary studies indicated that the N-terminal half of the ORF1 polyprotein of RHDV contains no protease. Thus, our data suggest that the nonstructural protein p16, like the 2A protein of hepatitis A virus, has no proteolytic activity, which implies that all cleavages in the viral polyprotein might be executed by the 3C-like TCP (48, 49). For poliovirus, experimental evidence which indicates a multifunctional role of

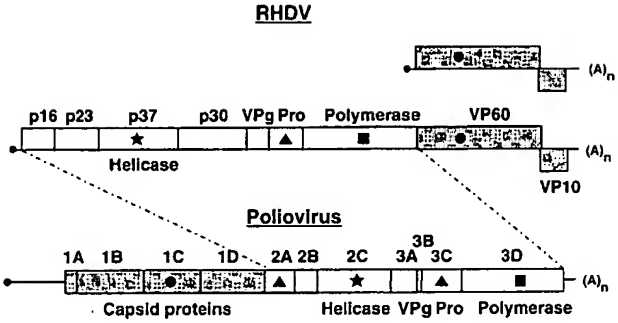

FIG. 10. Genetic map of RHDV in comparison with poliovirus. Schematic representations of the RHDV and poliovirus RNAs are shown, with noncoding regions shown as solid lines, coding regions drawn as bars, and VPgs indicated as small black circles. Nonstructural proteins are represented by open bars, and structural proteins are represented by shaded bars. Conserved amino acid sequences are indicated: ★, nucleotide-binding motif; ▲, cysteine protease; ■, polymerase domain; ●, region of VP60 homologous to 1C (VP3) of poliovirus.

the 2A protein in the replication cycle has been reported (18, 30, 34, 43). Some studies also suggest that the proteolytic activity of the 2A protein is dispensable for some of these functions (3). In view of these findings, it will be an interesting task for future studies to determine if the picornavirus 2A proteins and the amino-terminal cleavage product of the calicivirus polyprotein share a nonproteolytic function.

Our results concerning processing of the N-terminal part of the RHDV polyprotein differ considerably from the data published by Alonso and coworkers (1). Those authors found a protein of 80 kDa which probably encompasses the three processing products p16, p23, and p37 identified in the present study. The reason for this discrepancy is presently not known. For Southampton virus, a 41-kDa protein corresponding to p37 was identified, indicating that the amino-terminal part of the polyprotein of this calicivirus is processed in vitro (29). However, the same authors did not detect polypeptides corresponding to p16 or p23. Instead, a protein of 48 kDa was found, which again is apparently not further processed in vitro. Like RHDV, the human caliciviruses cannot be propagated in tissue culture, and further processing of p48 might occur within infected cells.

Cleavage of the poliovirus polyprotein gives rise to several long-lived precursor proteins that are only partially cleaved. Interestingly, some of these precursors composed of two final cleavage products are analogous to some cleavage products generated in vitro from the ORF1 polyprotein of RHDV. Most notable among these precursors are the 2BC, 3AB, and 3CD proteins of poliovirus, which correspond to the cleavage products p60, p41, and p69 of RHDV, respectively. Since each of the poliovirus precursors (3AB and 3CD [2, 14, 19, 20, 26, 33, 41, 42, 55] and 2BC [4, 9]) either has been shown or is presumed to have a functional role in genome replication distinct from its final products, it is interesting that the analogous fusion proteins are generated from the ORF1 polyprotein of RHDV, even though interpretation of these data has to take into account the limitations of the in vitro system.

A common feature of all caliciviruses is the existence of a small open reading frame of 300 to 600 nucleotides in length that overlaps the gene of the major capsid protein and extends close to the 3′ end of the genomic RNA (7, 8, 22, 27, 31, 37). Our results show that a capsid protein of approximately 10 kDa (VP10) is encoded in the respective open reading frame (ORF2) of RHDV. This protein was also observed after in vitro translation of RHDV virion RNA (data not shown). Interestingly, the apparent molecular mass of this basic protein is considerably lower than the calculated molecular mass of the ORF2 polypeptide (12.7 kDa). It is presently unclear whether this is due to either its amino acid composition or proteolytic cleavage of the primary translation product. In a recent study, the corresponding ORF3-encoded polypeptide of FCV has been identified in FCV-infected cells (21). Like VP10 of RHDV, this protein has an apparent molecular mass considerably lower than the calculated molecular mass of the ORF3-encoded polypeptide. Although VP10 was identified as a virion component for the first time in the present study, it is highly likely that the corresponding proteins of other caliciviruses also represent structural proteins. The function of the ORF2 polypeptide is not yet known, but it is intriguing that, according to our data for RHDV, this protein appears to be present in viral particles in substoichiometric amounts compared with the capsid protein VP60. Recently, it was shown that VP60 alone is able to assemble into virus-like empty particles that are indistinguishable from those which are found in livers of RHDV-infected animals (28, 35, 50). Considering these findings, we propose that VP10 could interact with both the major capsid protein VP60 and the viral RNA, thereby mediating specific encapsidation of the viral genome.

Within the picornavirus superfamily, caliciviruses are the only members which produce a subgenomic RNA. At least for RHDV, this RNA is efficiently packaged into virions, which is not a general feature of viral subgenomic RNAs. Furthermore, minus-strand copies of the subgenomic RNA have been detected in FCV-infected cells, suggesting that the subgenomic RNA is replicated like the genomic RNA (6). Taken together, these data indicate that the subgenomic RNAs of caliciviruses exhibit basic properties of a genome segment, namely, encapsidation and replication. Previous studies have demonstrated that RHDV makes use of two mechanisms to synthesize the capsid protein VP60 (5, 39, 50, 54). The first mechanism involves translation of the genomic RNA, which generates a precursor protein that is cleaved at the N-terminal boundary of the capsid protein. This translation strategy resembles those of picorna-, poty-, and sequiviruses, which are members of the picornavirus superfamily with monopartite genomes. Like RHDV, these viruses encode the capsid and the nonstructural proteins in a single open reading frame (11, 24, 45). The other expression mechanism, which is common to all caliciviruses, involves translation of the subgenomic RNA. This mechanism resembles the expression strategy of como- and nepoviruses, whose genomes are composed of two RNA molecules. The larger RNA (RNA 1) encodes the nonstructural proteins, whereas the smaller one (RNA 2) serves as mRNA for the capsid proteins. Considering their peculiar features, the subgenomic RNAs of caliciviruses can be regarded as additional genome segments that are used to express the structural proteins and in this respect represent the functional equivalent of the RNA 2 of como- and nepoviruses. Thus, the strategy of RHDV to express the major capsid protein can be best described as a unique combination of two known expression mechanisms that are used by other members of the picornavirus superfamily. These conclusions support the idea that caliciviruses or an ancestor with a similar genome organization and expression strategy constitute an evolutionary link between the two groups of viruses of the picornavirus superfamily with mono- or bipartite genomes.

ACKNOWLEDGMENTS

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